

**OPTIMIZING EMBRYO CULTURE CONDITIONS AND
SPENT CULTURE MEDIA ANALYSIS AS PREDICTORS OF
EMBRYO QUALITY AND PREGNANCY**

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**A dissertation submitted in fulfilment
of the requirements for the degree of
Philosophiae Doctor (PhD)**

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DECLARATION

Hereby I, the undersigned, declare that the thesis '*Optimizing embryo culture conditions and spent culture media analysis as predictors of embryo quality and pregnancy*' is my own work, that it has not been submitted previously for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Khalied Kaskar

Signed: _____

Date: 24 February 2021



DEDICATION

This work is dedicated to my wife, Shona, and daughters, Nina and Yasmin, for encouraging me to forge ahead and follow my dreams, and reminding me that one is never too old to learn.



ACKNOWLEDGMENTS

I would like to extend my most sincere gratitude and appreciation to the following people for their invaluable contributions to the successful completion of this work.

- My supervisor, Professor Ralf Henkel (Dept. of Medical Bioscience, University of the Western Cape, Bellville, South Africa), for his invaluable guidance, teaching and support.
- My co-supervisor and Medical Director, Dr. William Gibbons (Director of Reproductive Endocrinology and Infertility, Dept. of Obstetrics and Gynecology, Baylor College of Medicine, Houston, TX, USA) for affording me the opportunity to pursue my dream of obtaining a Ph.D. Your constant support, motivation, encouragement and constructive criticism is greatly appreciated.
- My lab director, Dr. Richard Cochran (Assistant Professor, Dept. of Obstetrics and Gynecology, Baylor College of Medicine, Houston, TX, USA), for your support and assistance.
- Dr. Chellakan Selvenesan Blesson (Assistant Professor, Dept. of Obstetrics and Gynecology, Baylor College of Medicine, Houston, TX, USA) for all your brilliant insights and advice.
- Dr. Kanaan Alpadi (Director of Scientific Affairs, Ansh Laboratories, Webster, TX, USA) and all the staff at Ansh Laboratories, for performing the GDF-9 analyses.
- Charles Dorsey (Senior Director of Diagnostics, Aytu Bioscience, Englewood, CO, USA) for providing the MiOXSYS® System and test kits to perform the oxidative stress studies.

- The best embryology team, Daneeka Hamilton, Amanda David, and Dr. Yana Kisarova (Family Fertility Center, Texas Children’s Hospital, Houston, TX, USA), for all your help with mouse embryos and time-lapse annotations, and for all your support and encouragement.
- Dr. Liubin Yang (Dept. of Obstetrics and Gynecology, Baylor College of Medicine, Houston, TX, USA) for helping with time-lapse annotations.
- Administrative teams of both Baylor College of Medicine and Texas Children’s Hospital, for allowing me to do these research projects.
- Dr. Ayub Pangarker, for his lifelong friendship, encouragement and support throughout the years, and for always being there for me both physically and “metaphysically”.
- My parents-in-law, Louis and Cynthia Grammer, for all their inspiration, love, support and encouragement.
- My parents, Abdulrehman and Rabiya, and brothers, Salim and Nabeal, for all their love, support and guidance throughout my life.
- My wife, Shona, and daughters, Nina and Yasmin, for all your love and for always supporting me through good times and bad, and being patient with me during this journey.

SCIENTIFIC ACHIEVEMENTS

The following parts of this thesis have been published or presented at institutional, national or international conferences. These are listed below, in addition to institutional and international scientific recognition.

Published conference proceedings

1. **Kaskar K**, Cochran R, Blesson C, Hamilton D, David A, Henkel R, Gibbons W (2019): Insulin and IgF-1 Does Not Alter the Morphokinetics of Mouse Embryo Development. *Reproductive Sciences*, 26 (Suppl 1), 196A.
2. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS (2019): Insulin and IgF-1 does not alter the morphokinetics of mouse embryo development. *Reproductive BioMedicine Online*, Volume 39, e12
3. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS (2019): Does culture of embryos in an ultra-low (2%) oxygen environment yield better blastocyst development than 6% oxygen using time-lapse morphokinetics? *Reproductive BioMedicine Online*, Volume 39, e9.
4. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS (2019): Finding a better mouse model for quality control and research studies in the IVF laboratory. *Reproductive BioMedicine Online*, Volume 39, e10 - e11

5. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS (2019): Increasing the efficacy of mouse embryo assays for quality control in the IVF laboratory. *Fert. Steril.* 112 (3), e271.
6. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS (2019): Does supplementation of media with insulin or insulin-like growth factor 1 (IGF-1) enhance morphokinetics of mouse embryo development? *Fert. Steril.* 112 (3), e271.
7. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS (2019): Effect of ultra-low oxygen (2%) environment on mouse embryo morphokinetics and blastocyst development. *Fert. Steril.* 112 (3), e270-e271.
8. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Alpadi K, Kumar A, Kalra B, Mistry S, Savjani G, Gibbons W, Blesson CS: Growth differentiation factor 9 (GDF-9) levels in spent culture media and pregnancy outcome. *Reproductive BioMedicine Online*, Volume 41, e4.

Poster presentations

1. **Kaskar K**, Cochran R, Blesson C, Hamilton D, David A, Henkel R, Gibbons W: Insulin and IgF-1 Does Not Alter the Morphokinetics of Mouse Embryo Development. The 66th Annual Scientific Meeting of the Society for Reproductive Investigation, Paris, France, March 12-16, 2019.

2. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS: Insulin and IgF-1 does not alter the morphokinetics of mouse embryo development. The 23rd Annual College of Reproductive Biology (CRB) Symposium, New Orleans, LA, May 16-18, 2019.
3. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS: Does culture of embryos in an ultra-low (2%) oxygen environment yield better blastocyst development than 6% oxygen using time-lapse morphokinetics? The 23rd Annual College of Reproductive Biology (CRB) Symposium, New Orleans, LA, May 16-18, 2019.
4. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS: Finding a better mouse model for quality control and research studies in the IVF laboratory. The 23rd Annual College of Reproductive Biology (CRB) Symposium, New Orleans, LA, May 16-18, 2019.
5. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS: Increasing the efficacy of mouse embryo assays for quality control in the IVF laboratory. The American Society for Reproductive Medicine (ASRM) Scientific Congress and Expo, Philadelphia, Pennsylvania, October 12 - 16, 2019.

6. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS: Does supplementation of media with insulin or insulin-like growth factor 1 (IGF-1) enhance morphokinetics of mouse embryo development? The American Society for Reproductive Medicine (ASRM) Scientific Congress and Expo, Philadelphia, Pennsylvania, October 12 - 16, 2019.

7. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Gibbons W, Blesson CS: Effect of ultra-low oxygen (2%) environment on mouse embryo morphokinetics and blastocyst development. The American Society for Reproductive Medicine (ASRM) Scientific Congress and Expo, Philadelphia, Pennsylvania, October 12 - 16, 2019.

Webinar presentations

1. **Kaskar K**, Cochran R, Hamilton D, David A, Henkel R, Alpadi K, Kumar A, Kalra B, Mistry S, Savjani G, Gibbons W, Blesson CS: Growth differentiation factor 9 (GDF-9) levels in spent culture media and pregnancy outcome. Selected abstract presented as a webinar for the College of Reproductive Biology (CRB) in conjunction with the International IVF Initiative (i3), May 12, 2020.

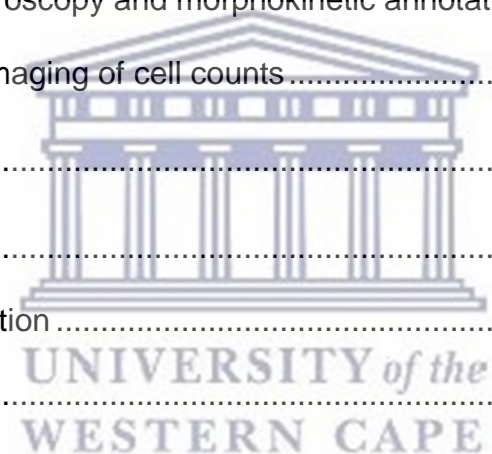
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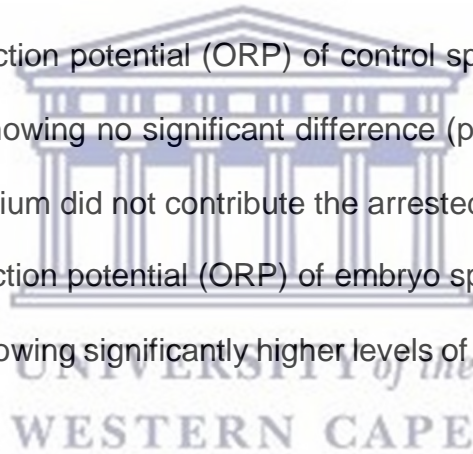
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ABSTRACT

The aim of this thesis is first, to evaluate various culture conditions to improve embryo development, and secondly, to analyze spent culture media for any biomarkers that may be predictive of embryo health.

Single-step and sequential culture media were compared in both Planer and EmbryoScope™ incubators. Single-step media resulted in better blastocyst development compared to sequential media and the EmbryoScope™ incubation system showed slight improvements in embryo development than the Planer system.

The benefits of supplementing the culture medium with either insulin or insulin-like growth factor 1 (IGF-1) or culturing in a 2% O₂ environment, using two different strains of mice (hybrid and C57), as well as the suitability of these strains for quality control were compared. In insulin, hybrid embryos were slower to blastulate and had a lower blastocyst rate, whereas C57 embryos were slower to the morula and faster to blastocyst stages, and lower blastocyst rate than the controls. IGF-1 showed no difference in time-lapse morphokinetics (TLM) or blastocyst rates compared to controls in both hybrid and C57 embryos. Under 2% O₂, hybrid embryos showed no significant difference in TLM up to the 8-cell stage, but slowed down afterwards, resulting in blastocysts with significantly lower cell counts than the 6% O₂ group. The C57 embryos were slower to reach morula and expanded blastocyst, and had lower blastocyst rates in 2%O₂ vs 6%O₂. The C57

strain had significant slower overall embryo development for all time points than hybrid embryos in insulin, IGF-1 and ultra-low O₂, as well as lower blastocyst rates.

Measurement of growth differentiation factor 9 (GDF-9) and oxidation-reduction potential (ORP) in spent media as markers for embryo health were evaluated. Day 5 human blastocysts yielded higher pregnancy rates and GDF-9 levels in spent media compared to Day 6 blastocysts, but TLM parameters showed no impact on pregnancy outcome. In Day 6 blastocysts, the non-pregnant group showed significantly faster embryo development compared to the clinically pregnant group up to the 8-cell stage and start of blastulation. GDF-9 did not show any significant differences between non-pregnant and pregnant groups of Day 5 or Day 6 embryo transfers. ORP in spent media from good quality Day 3 embryos that developed into blastocysts were significantly higher than from those that did not, with no difference in control medium ORP. Spent media from arrested embryos showed lower ORP than their corresponding controls. Arrested embryos had slower development at syngamy, morula, blastulation and blastocyst stages.

The single step medium in the EmbryoScope™ is the preferred choice for embryo culture. Insulin or IGF-1 media supplementation or 2% O₂ culture did not provide any benefit to embryo development. The C57 mouse strain is more sensitive and may be better to detect changes in culture conditions, and therefore better model for quality control assays. GDF-9 values decrease from Day 5 to Day 6 which gives new insight to understanding the role of GDF-9 during embryogenesis. ORP in spent media indicate that embryos that developed into blastocysts did not contribute to ROS, but maintained ORP balance.

KEYWORDS

Mouse embryo

Human embryo

Spent culture media

IVF

Insulin

Insulin-like growth factor-1 (IGF-1)

Low oxygen tension

Oxidation-reduction potential (ORP)

Antioxidant capacity

Growth differentiation factor-9 (GDF-9)

Time-lapse morphokinetics (TLM)

Infertility

Pregnancy



1. INTRODUCTION

1.1. General introduction

Assisted reproductive technologies (ART) have provided a solution for millions of infertile couples worldwide to have children of their own. Depending on the cause of infertility, various treatment options exist such as intra-uterine insemination (IUI), *in-vitro* fertilization (IVF) or in cases of severe male factor infertility, intra-cytoplasmic sperm injection (ICSI). For patients with advanced maternal age or recurrent pregnancy loss, embryos can be screened for chromosomal abnormalities using Preimplantation Genetic Testing for Aneuploidies (PGT-A) or for specific single gene disorders using Preimplantation Genetic Testing for Monogenic disorders (PGT-M), prior to transfer into the uterus.

Despite all these innovative techniques, there is still not a 100% success rate, leaving a significant number of couples childless even after several treatment attempts. These treatments could fail at different stages, such as ovarian stimulation, oocyte maturation, fertilization, embryo development, implantation, or maintaining a pregnancy as these events pose several challenges to embryologists and physicians and various research efforts are directed to improve techniques for each of these stages.

From a laboratory perspective, improvements are constantly investigated focusing on better methods for culture media supplementation and culture conditions to improve embryo development, and selection of the best embryo(s) to predict successful

implantation. Embryo selection has always relied on embryo morphology, and more recently in combination with PGT-A. Time-lapse morphokinetics (TLM) of embryos has also been proposed to help predict embryo implantation. However, there is still great discrepancy in the literature and the value of TLM has become controversial, possibly due to the lack of standardization of culture conditions among clinics. It appears that the beneficial use of TLM, if any, would be clinic-specific as each clinic would have to define their own parameters.

Improvements in culture media over the years have come about in the form of commercial production and testing. There are two culture systems available in the market today: (1) a sequential two-step system, and (2) a simplified one-step system. The benefits of the one-step system is that it allows for continuous undisturbed culture of embryos and eliminates the need to change the medium on Day 3 which is traditionally done with a two-step system to remove toxic ammonium buildup. One breakthrough that allowed for the reduction in ammonium buildup was the replacement of glutamine with L-glutamine in the medium, even though the detrimental effects of ammonia buildup has subsequently been disputed and shown to be unjustified. However, since almost all culture media used in IVF today are commercially obtained, the exact chemical composition of these media is unknown. Various chemicals and additives have been used to supplement media in efforts to improve embryo development.

Even though these media undergo stringent quality control testing (osmolarity, pH and mouse embryo assay) by the manufacturers, most IVF laboratories perform their own in-

house quality control prior to use, most notably using a mouse embryo assay (MEA). Mouse embryos have also been under scrutiny for their ability to detect subtle toxic substances that may affect human embryos. Since mouse embryos are hardier than human embryos, these often pass mouse embryo bioassays with >80% blastocyst development rates. In light of this, there have been studies to find more appropriate strains of mouse embryos that would be more compatible with human embryos and more sensitive to detect subtle changes in embryo culture conditions.

1.2. Culture medium and incubation systems

Proper culture conditions are the cornerstone of a good IVF lab. Many variables come into play such as type of medium, medium pH, temperature, osmolarity, air quality (Pool et al. 2012), individual or group culture, and type of incubators. The incubator is one of the most important components to embryo culture as it provides all the necessary conditions for a successful culture (Swain 2014).

Incubator types can be categorized into (1) CO₂ only, (2) low O₂ with inherent gas mixer, and (3) low O₂ using pre-mixed gas combinations. Regulation of gases, especially CO₂, is essential as it helps to maintain the pH of the culture medium, as this is buffered using a bicarbonate system. pH has been shown to impact gamete function and embryo development (Swain 2012). Decreased O₂ has also been found to help embryo development in both animals and humans (Bavister 2004, Bontekoe et al. 2012, Mantikou et al. 2013) and is especially important in extended culture to the blastocyst stage (Kovacic and Vlaisavljevic 2008, Meintjes et al. 2009, Waldenström et al. 2009).

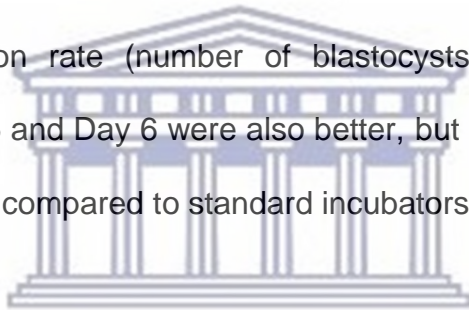
The major factor influencing a consistent gas supply is the frequent opening and closing of the incubator door to add or remove dishes. Based on the size of the incubator, the recovery time for gases to re-stabilize might vary. Traditional “large box” incubators, which have a chamber volume of ~150-200 L may need an extended time to refill compared to small box incubators, with chamber volumes ranging between ~14-48 L, the latter having shown to improve gas recovery, decreased environmental stress and enhance embryo development (Avery and Greve 1992).

Benchtop incubators have even further decreased the size of the incubation chamber to as little as ~0.31-0.5 L, allowing for improved gas recovery (Swain 2014). Of note, in our laboratory at the Family Fertility Center, we have used a combination of Planer BT37 incubators with an approximate chamber volume of 0.43 L and an EmbryoScope™ incubator which has a volume of 2.4 L. Benchtop incubators have been shown to improve culture environment recovery times compared to small box incubators (Fujiwara et al. 2007) and large box incubators (Lee et al. 2010). With regards to the EmbryoScope™ incubator, no differences were noted compared to standard large box incubators in terms of pronuclear formation (Hill et al. 2013), blastocyst formation, blastocyst viability or ongoing pregnancy rate (Cruz et al. 2011) or embryo development, implantation rate or clinical pregnancy rate (Kirkegaard et al. 2012).

Time-lapse incubation systems may assist with identifying embryos with high implantation potential and increase clinical outcomes (Wong et al. 2010, Aparicio et al. 2013, Chen et

al. 2013, Conaghan 2014, Kirkegaard et al. 2015). Some studies compared embryo quality between TLM and traditional incubators in the mouse (Pribenszky et al. 2010), bovine (Holm et al. 1998) and also in humans, although these were from donor cycles and not representative in the infertile population (Cruz et al. 2011).

Scioro et al. showed significant improvements in embryo quality on Day 3 in embryos cultured in the EmbryoScope™ time-lapse incubator compared to a benchtop incubator (Scioro et al. 2018). They also saw a higher proportion of good quality blastocysts on Day 5 in the EmbryoScope™, but these differences were not statistically significant. The overall blastocyst utilization rate (number of blastocysts suitable for transfer and cryopreservation) on Day 5 and Day 6 were also better, but not statistically significant in the EmbryoScope™ group compared to standard incubators.



The EmbryoScope™ has been shown to minimize variations of temperature and gas concentrations and has a fast recovery period to optimal conditions compared to large incubators (Meseguer et al. 2012). Furthermore, the EmbryoScope™ has a built-in active carbon filter to purify the air and remove volatile organic compounds (VOC) that can negatively impact embryo quality. It also reduces the number of door openings which have been shown to improve stability of culture conditions and increase embryo viability in mouse embryos (Gardner and Lane 1996) and increase blastocyst formation in humans (Zhang et al. 2010). Due to the limited exposure of embryos outside the incubator, the authors recommended the use of the EmbryoScope™ since there was comparable blastocyst development and improved cleavage stage development.

Maintaining humidity within the incubator is a way to reduce culture media evaporation and subsequent increases in osmolarity, which can negatively affect embryo development (Lane et al. 2008, Swain et al. 2012). However, humidity is not essential if the medium is overlaid with a layer of mineral oil (Swain 2014). While recommended ranges exist for many of the incubator variables (Wale and Gardner 2016), no optimal value for humidity during embryo culture has been determined yet (Cairo Consensus Group 2020).

Osmolarity is also an important consideration when preparing embryo culture dishes. It has been shown that the volume of the drop, stage temperature, and presence of air flow all affect osmolarity which can impair mouse embryo development (Swain et al. 2012). Ideal osmolarity for embryo development ranges from ~255-295 mOsm/kg, and osmolarities out of this range could impair embryo development (Brinster 1965, Hay-Schmidt 1993, Miyoshi et al. 1994, Liu and Foote 1996, Dawson et al. 1998, Richards et al. 2010). Even though commercial media are sold at a quality control (QC) acceptable osmolarity range, the manner in which the dishes are prepared could affect the osmolarity prior to culture. Compared to culture media straight out of the bottle, media prepared in 10 μ L microdrops at 37°C with air flow showed a significant increase in osmolarity of ~40 mOsm/kg, placing it well outside the recommended range for embryo culture (Swain 2012). Furthermore, when comparing embryo cultures in media of increasing osmolarities, significant decreases in embryo development were noted in the 8-cell stage, early blastocyst, total blastocyst, expanded/hatching blastocyst and hatched blastocyst stages using F1 hybrid 1-cell mouse embryos (Swain et al. 2012).

There are two main culture systems: (1) single system from insemination to Day 5/6 (Biggers 1998, Biggers 2002, Biggers and Summers 2008), and (2) a sequential system with a changeover of media on Day 3 (Gardner et al. 2002). The Cairo Consensus Group concluded that both single-step and sequential media can support embryo development and high outcomes and that the current data is limited and insufficient to determine if one is superior to the other (Cairo Consensus Group 2020). It has also been concluded by a recent Cochrane review (Youssef et al. 2015) and others (Sfontouris et al. 2016, Dieamant et al. 2017) that there are no differences in outcomes between these two culture systems.



It is imperative that each IVF laboratory perform its own quality control to determine the best culture system specific to its own conditions to provide the best embryo quality. Due to the conflicting reports regarding the benefits of sequential versus single step culture medium systems, this thesis aims to compare these two systems in terms of their ability to maintain embryo development up to the blastocyst stage. Furthermore, due to the various incubators commercially available on the market today, most notably the time-lapse system (EmbryoScope™) and traditional benchtop incubators (Planer), it is important to determine which one would be better for embryo culture.

1.3. Media supplementation with insulin or insulin-like growth factor 1 (IGF-1)

Even with different formulations of culture media from various manufacturers, the basic ingredients are essentially the same, such as glucose, pyruvate, and amino acids, but various medium supplements have been proposed to improve embryo development (Cairo Consensus Group 2020). Growth factors have been shown to be involved in cellular proliferation, differentiation and morphogenesis during mammalian embryogenesis (Hill 1992, Schultz and Heyner 1993). Of these, insulin and insulin-like growth factor-1 (IGF-1) play an important role in cellular differentiation (Herrler et al. 1998). Both of these support early embryo development by protecting the embryo from apoptosis and increasing cell proliferation (Herrler et al. 1998). Other growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF) have also been used to supplement culture media but a recent Cochrane review concluded that it does not show any benefit to pregnancy outcome (Armstrong et al. 2020).

IGF-1 is a small peptide of 70 amino acids with a molecular mass of 7,649 daltons (Laron 2001, Velazquez et al. 2012) and is secreted by the liver as well as granulosa cells (Hernandez et al. 1988, Giudice 1992, Arany et al. 1994). It plays several roles in follicular and embryo development (Adashi et al. 1985, Guzeloglu-Kayisli et al. 2009) and is involved in regulating cellular proliferation, differentiation and survival (Benito et al. 1996, Vincent and Feldman 2002), as well as having mitogenic and anti-apoptotic effects (Velazquez et al. 2009, Velazquez et al. 2011). However, in both cattle and humans, oocytes from follicles with high IGF-1 levels had an impaired *in vitro* developmental

potential to form blastocysts (Wang et al. 2006, Siddiqui et al. 2009). In contrast, Velazquez et al. showed that short *in vivo* exposure of bovine oocyte to a supraphysiological IGF-1 environment increased inner cell mass (ICM) proliferation from morula to blastocyst development (Velazquez et al. 2012). IGF-1 and its receptor have been shown to be present from the oocyte stage throughout preimplantation (Inzunza et al. 2010). IGF-1 has been shown to increase blastocyst cell number, especially in the inner cell mass (Harvey and Kaye 1992, Lighten et al. 1998, Brison 2000, Markham and Kaye 2003, Behr and Wang 2004, Kim et al. 2005) with a 25% increase in blastocyst formation rate and 59% increase in ICM cells (Lighten et al. 1998). Adding IGF-1 to the culture medium have shown a significant reduction in the percentage of apoptotic nuclei in the blastocyst by 50% in humans (Lighten et al. 1998, Spanos et al. 2000, Behr and Wang 2004) and in mice (Makarevich and Markkula 2002). In mouse models, supplementary IGF-1 showed an anti-apoptotic effect with a resulting increase in blastocyst formation rate (Lin et al. 2003). However, some reports show no benefit to adding IGF-1 to the medium in terms of development rate (Paria and Dey 1990, Heyner et al. 1993). Furthermore, other studies have shown that high levels of IGF-1 are embryotoxic (Katagiri et al. 1996, Katagiri et al. 1997, Chi et al. 2000, Green and Day 2013, Irani et al. 2018). High concentrations are thought to decrease the expression of IGF-1 receptors and reduce insulin-stimulated glucose transport in the blastocyst, thereby triggering apoptosis, leading to pregnancy loss (Jurisicova et al. 1996, Chi et al. 2000, Hardy and Spanos 2002, Irani et al. 2018). After transferring euploid blastocysts, it has been reported that women with high serum levels of IGF-1 have a higher risk of pregnancy loss compared to those with low levels (Irani et al. 2018).

Insulin is needed for growing cells in culture and when added to the culture medium for mouse embryos, has been shown to stimulate amino-acid transport (Kaye et al. 1986), protein synthesis (Harvey and Kaye 1988) and to improve embryo development (Harvey and Kaye 1990, Gardner and Kaye 1991). In addition, insulin also increases the cell number of the inner cell mass of mouse embryos and it was suggested that this increase is mediated via insulin receptors that are functionally expressed at the time of compaction at the 8-cell stage (Harvey and Kaye 1990). Insulin has also been shown to increase cell proliferation and decrease apoptosis in *in vitro* cultured embryos (Herrler et al. 1998). Furthermore, when insulin was absent from the culture medium, a decrease in embryo development and cell proliferation was noted (Harvey and Kaye 1990, Gardner and Kaye 1991, Kaye and Gardner 1999) indicating that embryo growth is dependent on insulin. In a recent study, addition of insulin to the culture medium resulted in a higher proportion of high quality Day 3 embryos, higher rate of compaction on day 3, increased blastocyst formation as well as improved blastocyst quality (Fawzy et al. 2017). Furthermore, the authors found a positive correlation between insulin supplementation and clinical pregnancy rates (Fawzy et al. 2017).

In mice, insulin and IGF-1 receptors have been found in embryos from the 8-cell stage and beyond as well as in trophectoderm cells (Heyner et al. 1989, Heyner et al. 1989, Herrler et al. 1997). Insulin and IGF-1 bind to these receptors from the morula stage in both mice (Mattson et al. 1988) and rabbits (Herrler et al. 1997). Insulin and IGF-1 increase embryo metabolism and cell proliferation by increasing glucose uptake (Kaye et

al. 1992, Pantaleon and Kaye 1996) and protein synthesis (Heyner et al. 1989, Doherty et al. 1994).

Not only does IGF-1 increase the cell numbers in the blastocyst in general (Doherty et al. 1994) but also more specifically in the inner cell mass (Harvey and Kaye 1992). IGF-1 has been shown to increase the number of blastocysts resulting from morulae by a factor of 2 to 3, as well as increasing embryo diameter and cell proliferation (Herrler et al. 1998). It is unclear if the embryo itself produces IGF-1 since some have detected it in oocytes up to the blastocyst stage (Watson et al. 1992, Doherty et al. 1994, Inzunza et al. 2010, Green and Day 2013), while others have not (Lighten et al. 1997). It is present, however, in the oviduct (Carlsson et al. 1993, Dalton et al. 1994, Schmidt et al. 1994) and the uterus (Murphy et al. 1987, Geisert et al. 1991, Ko et al. 1991, Stevenson et al. 1994). Furthermore, it has also been shown that rat embryos cultured in the absence of insulin resulted in a decrease in embryo growth and replenishment of insulin with as little as 10 ng/mL, restored embryo growth (Travers et al. 1989, Travers et al. 1992)

Group culture of embryos could also play a role in the embryo response to IGF-1 as this has been shown to improve development (Harvey and Kaye 1992, Markham and Kaye 2003). High density culture of mouse embryos (10-15 embryos with 1 embryo per microliter) showed significantly higher blastocyst development and hatching rates than individual culture (Green and Day 2013). This could be due to the presence of embryo derived growth factors that are beneficial to embryo development (O'Neill 1998).

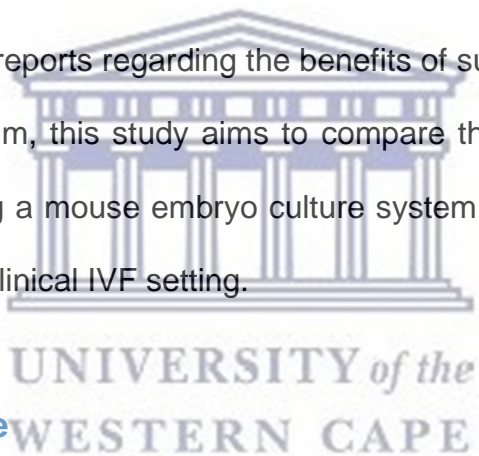
Furthermore, addition of exogenous IGF-1 in the culture medium resulted in an increased blastocyst development, but no effect on hatching rates (Green and Day 2013).

Apoptosis occurs during embryo development (Brison and Schultz 1997) and is increased during *in vitro* culture due to culture conditions that may be suboptimal to natural environments (Pantaleon and Kaye 1996, Jurisicova et al. 1998, O'Neill 1998, Kamjoo et al. 2002). IGF-1 plays an important role in preventing apoptosis during early embryo development (Lin et al. 2003) due to inhibition of caspase-3-like activation and Bcl-2 and Bax expression (Tamatani et al. 1998). In a study where apoptosis was artificially induced by ultraviolet light, addition of insulin or IGF-1 to the culture medium of UV-irradiated rabbit embryos showed significant improvements in blastocyst formation, showing that insulin and IGF-1 act as survival factors (Herrler et al. 1998). Since apoptosis may be caused by reactive oxygen species (ROS) (Joshi 1989, Costanzo et al. 1995, Verhaegen et al. 1995), insulin and IGF-1 is thought to oppose apoptosis by inhibiting ROS generation (Hockenbery et al. 1993, Kane et al. 1993). IGF-1 has been reported to possess antioxidative properties (Tilly 1996).

In terms of *in vitro* maturation, addition of IGF-1 to maturation medium has also been studied since there is evidence that growth factors in follicular fluid may help regulate oocyte maturation (Hsieh et al. 2009, Zamah et al. 2010). IGF-1 in ovarian and follicular fluid as well as IGF-1 receptors in the oocytes have been found (Danforth 1995). Some reports show that IGF-1 increases maturation rates of bovine (Lorenzo et al. 1994, Rieger et al. 1998) and porcine oocytes (Xia et al. 1994), while others have seen no influence in

bovine (Guler et al. 2000) and porcine oocytes (Reed et al. 1993). Porcine oocytes with cumulus cells have a higher capacity to mature than denuded oocytes (Sirotkin et al. 1998). Furthermore, in oocytes with or without cumulus, addition of 10 or 100 ng/mL IGF-1 stimulated nuclear maturation in both groups but higher doses (1000 ng/mL) had no effect (Sirotkin et al. 1998). The fact that IGF-1 could maintain the maturation rate in the absence of follicular or cumulus cells indicates that it may be a good substitute. Moreover, IGF-1 appears to act directly in the oocyte and is not mediated by secretions of surrounding follicular cells (Sirotkin et al. 1998).

Since there are conflicting reports regarding the benefits of supplementation of insulin or IGF-1 to the culture medium, this study aims to compare these two growth factors on embryo development using a mouse embryo culture system to determine if it would be advantageous to use in a clinical IVF setting.

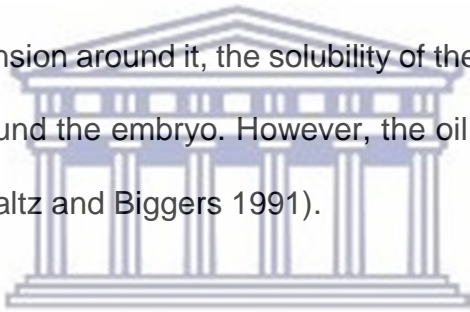


1.4. Low oxygen culture

During embryo culture, various conditions have been optimized to mimic the *in vivo* environment such as temperature, pH, and oxygen tension to enhance embryo development. The oxygen concentration in the reproductive tract in many species, such as rats, hamsters, rabbits, monkeys and humans, has been reported to be 2-8% (Mastroianni and Jones 1965, Yedwab et al. 1976, Kigawa 1981, Kaufman and Mitchell 1990, Fischer and Bavister 1993). Most specifically, oxygen tension in the monkey and human uterus has been shown to be about 1.5% (Yedwab et al. 1976, Fischer et al. 1992). Preovulatory follicular fluid contains an oxygen concentration ranging from 1-5.5%

surrounding the oocytes (Gosden and Byatt-Smith 1986). In addition, excessive oxygen is thought to be detrimental to early embryos possibly due to the oxygen-derived free radicals (Johnson and Nasr-Esfahani 1994, Guerin et al. 2001).

The embryo is surrounded by oviductal or uterine fluid from which it obtains various nutrients and oxygen in order to develop. Early embryos during their initial cell divisions use oxidative phosphorylation before compaction (Figure 1) which changes to aerobic glycolysis after compaction, when oxygen consumption increases significantly (Leese 1995, Martin 2000). *In vitro*, the embryo obtains oxygen through passive diffusion that is controlled by the oxygen tension around it, the solubility of the oxygen within the medium, and the zona pellucida around the embryo. However, the oil overlay does not appear to limit the rate of diffusion (Baltz and Biggers 1991).



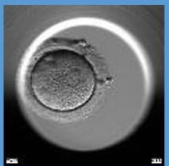
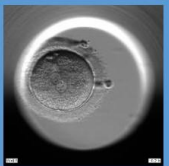
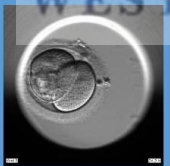
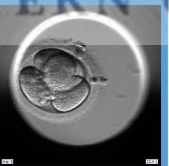
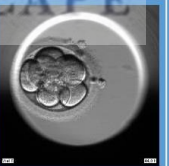
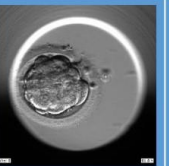
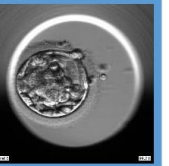
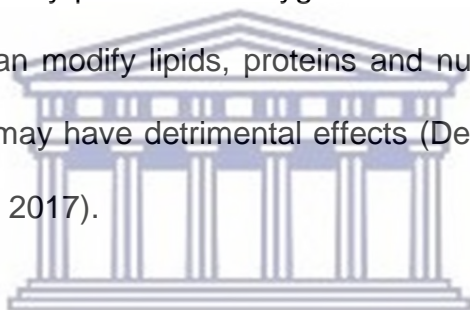
OXIDATIVE PHOSPHORYLATION				AEROBIC GLYCOLYSIS		
DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	
						
Mature oocyte	Fertilized oocyte	2 cell embryo	4 cell embryo	8 cell embryo	Morula	Blastocyst

Figure 1. Metabolic requirements of the embryos, showing a change in oxygen tension at time of compaction would be consistent with a general shift in the metabolic strategy of the embryo at time of embryonic genome activation. Modified from Morin (2017).

Another concern about culturing at higher oxygen levels is that it can increase the formation of reactive oxygen species (ROS). ROS production can be endogenous as a by-product of aerobic metabolism (Bielski and Arudi 1983, Kovacic 2012) or through exogenous sources such as follicular fluid, tubal fluid or the culture medium. Culture media have been shown to generate ROS at a higher rate than follicular fluid (Martin-Romero et al. 2008). Different types of culture media have been shown to have an inherent oxidation-reduction potential, which could expose the embryos to higher oxidative stress (Panner Selvam et al. 2018).

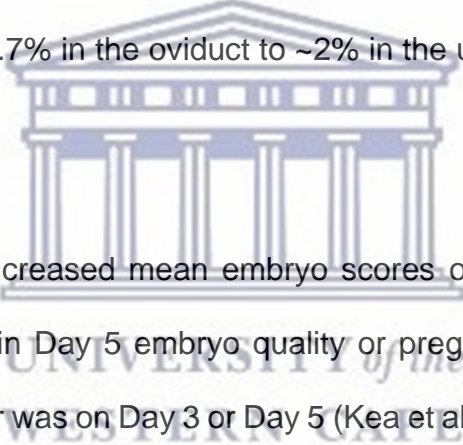
Reducing the O₂ level to 5% has been shown to be better than atmospheric oxygen in terms of embryo and blastocyst development, leading to the first IVF baby (Edwards et al. 1970, Steptoe et al. 1971, Steptoe and Edwards 1978). Other studies showed that 5% O₂ had better clinical pregnancy rate, live births and implantation (Meintjes et al. 2009, Bontekoe et al. 2012, Nastri et al. 2016). Furthermore, TLM studies have shown detrimental effects of atmospheric O₂ on mouse embryos where the first, second and third cleavage cycles were delayed compared to 5% O₂ (Wale and Gardner 2010). Similar delays in development was also noted in the third cleavage cycle of human embryos cultured in atmospheric oxygen compared to 5% O₂ (Wale and Gardner 2010, Kirkegaard et al. 2013). The Cairo Consensus Group (2020) showed that there is overwhelming evidence to support 5% O₂ for embryo culture but very little studies have looked into the health of children born from embryos cultured with 5% O₂ versus 20% O₂.

When comparing the culture of embryos in low oxygen environments with a concentration of 20% oxygen, reports have shown better embryo quality on Day 2 and Day 3 in terms of increased blastomere number and decreased fragmentation when using the low oxygen concentration (de los Santos et al. 2013). However, the authors did not find any significant differences in ongoing pregnancy rates per cycle and per transfer when using embryos cultured in 6% O₂ versus 20% O₂. Their study was limited to Day 3 culture as the end point and no data regarding blastocyst development was provided. Even though oxygen is necessary for embryo development, it also could be detrimental due to the formation of ROS as natural by-products of oxygen metabolism (Ufer and Wang 2011). These ROS metabolites can modify lipids, proteins and nucleic acids by altering their biological functions which may have detrimental effects (Dennerly 2007, Dennerly 2010, Ufer et al. 2010, Leite et al. 2017).



Low oxygen has a positive effect on embryo metabolism of glucose and enhances blastocyst development and pregnancy outcome (Meintjes et al. 2009, Waldenström et al. 2009, Bontekoe et al. 2012, Kovacic 2012). Lowering the O₂ from 20% to 5% has also been shown to improve embryo quality in Day 2, Day 3 and Day 5 embryos, as well as a 10% improvement in blastocyst development rate in both IVF and ICSI cases (Kovacic and Vlaisavljevic 2008). However, despite the improvement in embryo quality and the increased blastocyst formation rate, there were no significant differences in clinical pregnancy and implantation rates. Other studies have also shown no difference in embryo development by reducing the oxygen tension from 20% to 5% using 1 mL of medium without any oil overlay (Dumoulin et al. 1995). These authors further went on to study the

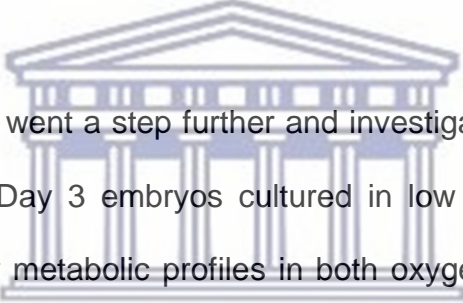
effects in 20 µl microdrops under oil which is more commonly used in IVF and found that lower O₂ tension still did not have any effect on Day 2 and Day 3 embryos scores nor on the pregnancy rate (Dumoulin et al. 1999). However, when culturing surplus embryos to Day 5, the low oxygen culture yielded higher blastocyst formation and an increased number of cells per blastocyst on both Day 5 and Day 6. Since the embryo transfers were still done on Day 2 and Day 3, it is not surprising that these authors did not see a difference in pregnancy rate. Preliminary studies showed that by lowering the oxygen tension from Day 3 to Day 5, there was an improvement in embryo cleavage and pregnancy rates (Kim et al. 2005). This would support early work showing that the oxygen tension decreases from 5-8.7% in the oviduct to ~2% in the uterus (Fischer and Bavister 1993).



Others have also shown increased mean embryo scores on Day 3 under low oxygen conditions, but no change in Day 5 embryo quality or pregnancy rates, irrespective of whether the embryo transfer was on Day 3 or Day 5 (Kea et al. 2007). However, this study utilized desiccators with 5% O₂ inside the 20% O₂ incubators as opposed to dedicated low oxygen incubators. Low oxygen culture has been reported to result in improved Day 3 embryo quality, Day 5 blastocyst quality and Day 6 blastocyst yield (Ciray et al. 2009) as well as increased pregnancy rates and live birth rates (Waldenström et al. 2009).

Meintjies et al. (2009) reported on embryos transferred on both Day 3 and Day 5 in lower oxygen conditions and found no differences in the implantation rate, live-birth implantation rate, clinical pregnancy rate nor live-birth rate in Day 3 transfers. In Day 5 transfers,

however, the implantation rate, live-birth implantation rate and clinical pregnancy rates were all significantly improved in the lower oxygen group compared to atmospheric oxygen. The live birth rate was also improved but failed to reach statistical significance ($p=0.087$). This clearly shows that there is a benefit to growing embryos in low oxygen from Day 3 to Day 5 in order to help blastocyst development. However, other reports showed no significant differences in the embryo quality scores on Day 3 and Day 5, blastulation rate, chemical pregnancy rate, clinical pregnancy rate or implantation rate when comparing embryos cultured in low oxygen and atmospheric oxygen during Day 3 to Day 5 (Nanassy et al. 2010).



De los Santos et al. (2015) went a step further and investigated the metabolic profile of spent culture media from Day 3 embryos cultured in low oxygen (6%) compared to atmospheric (20%). Similar metabolic profiles in both oxygen cultures conditions were found and no changes in glucose or fatty acids in the spent medium were seen. Since there were no differences in embryo quality on Day 3, the authors concluded that low oxygen does not affect the global metabolism of cleavage stage embryos. One limitation of this study was that embryos were only cultured to Day 3 and not to the blastocyst stage (Day 5).

Most of the low oxygen studies have focused on reducing the oxygen tension to either 5% or 6%. The mean peri- and postovulatory uterine oxygen concentration in humans is approximately 2% (Yedwab et al. 1976, Ottosen et al. 2006) and for the duration of the first trimester, the uterus is hypoxic (Rodesch et al. 1992). Kaser et al (2018) designed a

study to culture donated 2PN and 3PN embryos in 5% O₂ from Day 1 to Day 3 and then from Day 3 to Day 5 in either 5% O₂ or 2% O₂. At Day 3, there was no difference in good quality embryos prior to culturing them in 2% O₂. Embryos in the 2% O₂ group were less likely to arrest on Day 3 and more likely to reach the blastocyst stage. They found an increase in usable blastocysts in the 2% O₂ group, but fewer cells in these blastocysts. Interestingly, they also showed a decrease in amino acids, which could relate to an increased uptake in these embryos. Using RNAseq, Morin et al (2018) showed significant differences in gene expression between trophectoderm cells (TE) and inner cell mass (ICM) of frozen-thawed aneuploid blastocysts, but no differences in gene expression between embryos cultured at 2% O₂ versus 5% O₂ in either the TE or ICM. They concluded that ultra-low oxygen culture does not cause significant changes in expression of genes associated with mitochondrial function. However, bovine embryos showed significantly increased number of both ICM and TE cells in 5% O₂ compared to 20% O₂ (Leite et al. 2017). Mouse embryos cultured in 3% O₂ have higher blastocyst rates than those in 20% O₂, indicating that low oxygen usage could possibly benefit embryo viability by increasing antioxidant expression (Ma et al. 2017, Belli et al. 2020).

It is very clear from the literature that reducing the oxygen tension from atmospheric (20%) to 5-6% O₂ has beneficial effects on embryo quality and pregnancy rates. Most IVF laboratories have adopted the low oxygen culture environment. However, apart from the work done by Kaser et al. (2018) and Morin et al (2018), there is very little work done on the effects of reducing the oxygen tension even further to 2% which would mimic the

environment in the uterus. Furthermore, there have been no studies on time-lapse morphokinetics on the behavior of embryo development in ultra-low oxygen conditions.

Due to the lack of extensive research on ultra-low O₂ culture, this thesis aim to compare embryo development in 2% O₂ and conventional 6% O₂ environments as well as time-lapse morphokinetics using a mouse embryo culture system.

1.5. Strain of mouse embryos in quality control

Quality control (QC) is an essential part of human IVF and is also a requirement for laboratory accreditation (Gardner et al. 2005) and also a practice guideline from the Practice Committee of the American Society of Reproductive Medicine (2008).

There are several tests and assays that can be used to test culture media and contact materials for toxicity such as osmolarity, pH, endotoxin, human sperm survival, and the mouse embryo assay (MEA). Since most laboratory products, including culture media, are commercially obtained, they undergo QC from the manufacturer before their release. Even though beneficial effects of mouse embryo bioassays for QC testing in IVF labs have been reported (Ackerman et al. 1984, Quinn et al. 1984, Fleming et al. 1987), there is also criticism that it is both irrelevant and ineffective (Davidson et al. 1988, George et al. 1989, Fleetham et al. 1993). Culture of 2-cell mouse embryos to reach the blastocyst stage has been used as a standard bioassay for evaluating culture medium quality (Ackerman et al. 1984, Ackerman et al. 1985, Parinaud et al. 1987). However, there has been growing concern about the type of mouse embryo used in these assays in terms of

cell stage and mouse strain as these could yield different outcomes (Khan et al. 2013). Two- to four-cell mouse embryos are able to develop into blastocysts in media made with tap water, even in the absence of protein supplementation (Silverman et al. 1987, George et al. 1989). MEAs using 1-cell mouse embryos have been shown to be more sensitive than using 2-cell embryos (Davidson et al. 1988, Fleetham et al. 1993, Scott et al. 1993, Hughes et al. 2010, Morbeck et al. 2010).

The strain of mouse embryos could be a significant factor that could affect the sensitivity of these assays (Dandekar and Glass 1987, Chatot et al. 1990, Scott et al. 1993). Strains are usually from genetically identical inbred mice, genetically diverse outbred mice, and hybrid mice. The most commonly used is the hybrid strain since it is not prone to 2-cell block and produce embryos that have high blastulation rate (Suzuki et al. 1996), but this is not as important now since newer culture media are optimized for cleavage stage embryo culture. Fleetman et al (1993) compared two different strains of mouse embryos and found that the CD1 outbred embryos at the 2-cell stage did not show any impairment of blastocyst development (>75%) in media with different levels of endotoxins. Endotoxin levels as little as 1 ng/mL have been shown to be toxic to human embryos (Fishel et al. 1988), making this 2-cell mouse strain a poor choice for quality control testing. In contrast, one-cell embryos from inbred F1 hybrid mouse strain did show significantly lower blastocyst development rates in media with endotoxins (Fleetham et al. 1993). In contrast, when comparing the ability of two different strains of mouse oocytes to fertilize and reach the morula and blastocyst stages after exposure to various environmental stresses present during human oocyte retrieval, the CD1 mice did much better in reacting to

external stress than the F1 hybrid model (Jackson and Kiessling 1989). Comparing one-cell and two-cell embryos from three different strains of mice (CF1, Dub:(ICR), and CFW), in five different types of culture media, Dandekar and Glass (1987) showed that one cell mouse embryos were more sensitive than two-cell embryos and that the mouse strain used is an important factor in quality control bioassays.

From our experience in the IVF laboratory in the Family Fertility Center, we have observed that mouse embryo assays using 2-cell frozen-thawed hybrid mouse embryos usually yield >90% blastocyst development rate in both in house QC tests as well as proficiency testing for an outside testing agency such as American Association on Bioanalysts (unpublished data). These embryos tend to be hardy and due to their high blastocyst development rate, there is concern whether they would be sensitive enough to detect subtle changes in culture media or conditions that would otherwise be detrimental to human embryos in the IVF lab. Morbeck et al. have shown that mineral oil, which is routinely used in IVF culture passed the manufacturer's QC but had adverse effects on embryo culture and were subsequently recalled (Morbeck 2012, Morbeck et al. 2012). According to Khan et al. (2013), outbred mice were sensitive to culture stress in terms of having decreased blastocyst development than inbred or hybrid strains. It has been recommended that companies start using outbred mice as their final QC assay before releasing their products to the market (Khan et al. 2013).

Since blastocyst development itself is not always associated with viability (Lane and Gardner 1997), it has been proposed to incorporate time-lapse morphokinetics (TLM) to

the MEA (Wolff et al. 2013). TLM has been suggested as a predictor of viable human embryos for transfer (Gonzales et al. 1995, Arav et al. 2008, Lemmen et al. 2008). Even though several studies have promoted the use of cell division timing to select the best embryo for transfer (Pribenszky et al. 2010, Wong et al. 2010, Azzarello et al. 2012, Cruz et al. 2012, Hlinka et al. 2012, Kirkegaard et al. 2012, Meseguer et al. 2012), this has not been adequately defined for mouse embryos with respect to quality control studies. Using TLM, Wolff et al (2013) was able to show changes in cell division timings using TLM in toxic environments that were not apparent in blastocyst development rate.

It is clear from the literature that mouse strain plays an important role in the outcome of mouse embryo assays for quality control, but there is no clear consensus as to which strain is most optimal to use. The aim of this study is to compare two different strains of mouse embryos, namely a hybrid model and a C57 inbred model, to determine their efficacy in detecting subtle changes in embryo culture as measured by their ability to develop into blastocysts as well as their time-lapse morphokinetics.

1.6. GDF-9 in spent media and pregnancy outcome

Oocyte quality is an important limiting factor in female infertility (Gilchrist et al. 2008). There is recent evidence to show that the oocyte itself is a major regulator of follicular function and plays a critical role in the regulation of oogenesis, ovulation and fecundity (Eppig 2001, Gilchrist et al. 2004, McNatty et al. 2004, Gilchrist and Thompson 2007, Gilchrist et al. 2008). The oocyte secretes soluble growth factors, which act on

neighboring follicular cells to regulate various granulosa cell (GC) and cumulus cell (CC) function (Gilchrist et al. 2008).

Two of these oocyte-secreted growth factors, growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP 15), have been shown to play an important role since the absence of these causes sterility (Dong et al. 1996, Galloway et al. 2000). BMP-15 and GDF-9 are synthesized as 249-295 amino acid N-terminal pro-domains and 125-139 amino acid mature domains. The mature domains of GDF-9 and BMP-15 form 40 kDa and 34 kDa homodimers, respectively, and 37 kDa heterodimer (Pulkki et al. 2012, Peng et al. 2013). These two factors are members of the transforming growth factor β (TGF β) superfamily and play key roles in promoting follicle growth, maturation and oocyte competence (McNatty et al. 2005, Peng et al. 2013, Mottershead et al. 2015, Convissar et al. 2017), and are also central regulators of GC/CC differentiation, potential contraceptive targets. Further, these factors may be associated with ovarian dysfunction (Gilchrist et al. 2004, Shimasaki et al. 2004, Juengel and McNatty 2005, McNatty et al. 2007). The effects of these factors on folliculogenesis are illustrated in Figure 2. Oocyte competence is achieved through a variety of molecular, biochemical and morphological changes (McNatty et al. 2007).

GDF-9 and BMP-15 are important factors in the transition of the primordial follicle to the primary follicle stage (Figure 3). Granulosa cells (GC) help defend against ROS and by contributing to the oocyte's antioxidant defense also provide nutrients through glycolysis and cholesterol biosynthesis, all of which are regulated by GDF-9 and BMP-15. The

importance of GDF-9 and BMP-15 are important factors in regulating the function of GCs, GC communication, cumulus oocyte complex (COC) expansion and hyaluron production leading to ovulation, and inhibiting of progesterone production (Sanfins et al. 2018).

GDF-9 is required for female fertility since it has been shown that carriers with mutations in GDF-9 are sterile due to a block at the primary stage of folliculogenesis (Dong et al. 1996, Hanrahan et al. 2004, Gilchrist et al. 2008). In humans, aberrant expression of GDF-9 may be associated with polycystic ovarian syndrome (Teixeira Filho et al. 2002, de Resende et al. 2012). Rare mutations in both GDF-9 and BMP15 may cause premature ovarian failure (Di Pasquale et al. 2004, Dixit et al. 2006, Laissue et al. 2006, Simpson et al. 2014, Bouilly et al. 2016) and mutations on GDF-9 alone are associated with dizygotic twinning (Montgomery et al. 2004, Palmer et al. 2006). It has also been shown that anti-Mullerian hormone (AMH) production is regulated by oocyte-secreted factors in primary human cumulus cells and the combination of GDF9 and BMP15 potently stimulates AMH expression (Hussein et al. 2006).

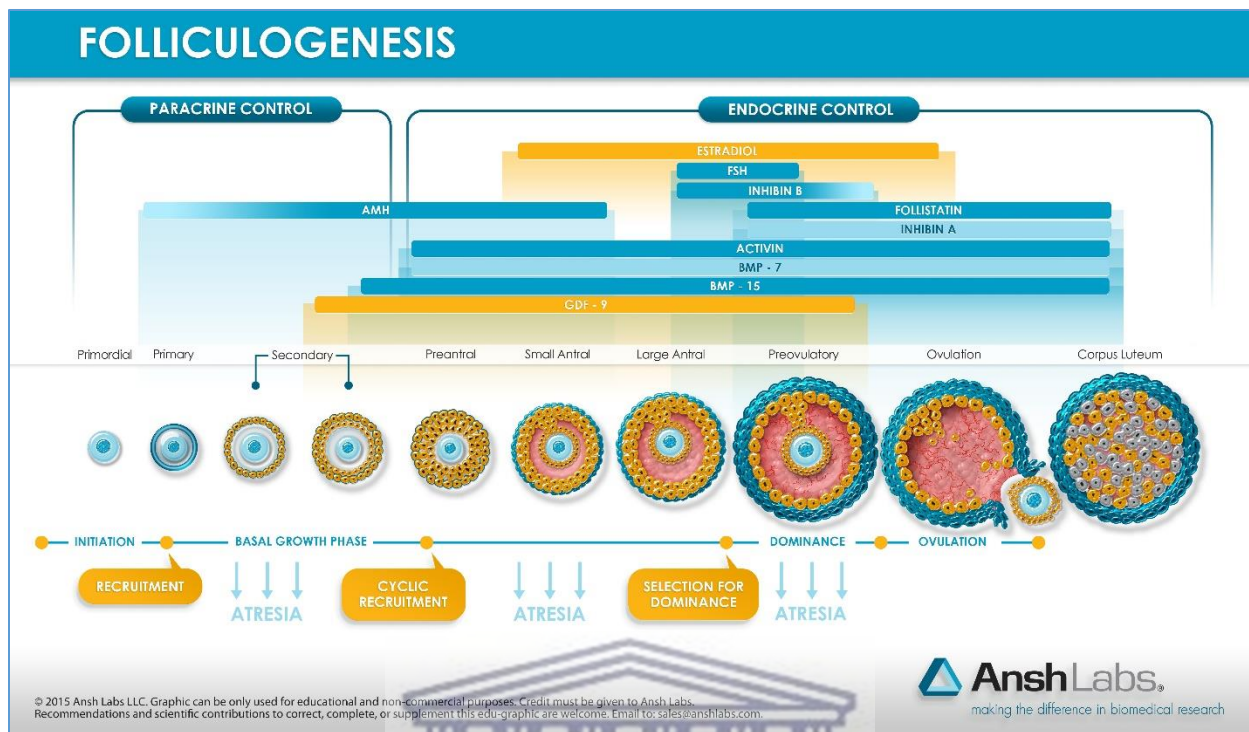


Figure 2. Schematic illustration of the effects of hormones and growth factors (GDF-9 and BMP-15) on the process of folliculogenesis. Reprinted with permission from Ansh Labs, Webster, TX, USA (<https://www.anshlabs.com/resources/folliculogenesis-educational-graphic/>)

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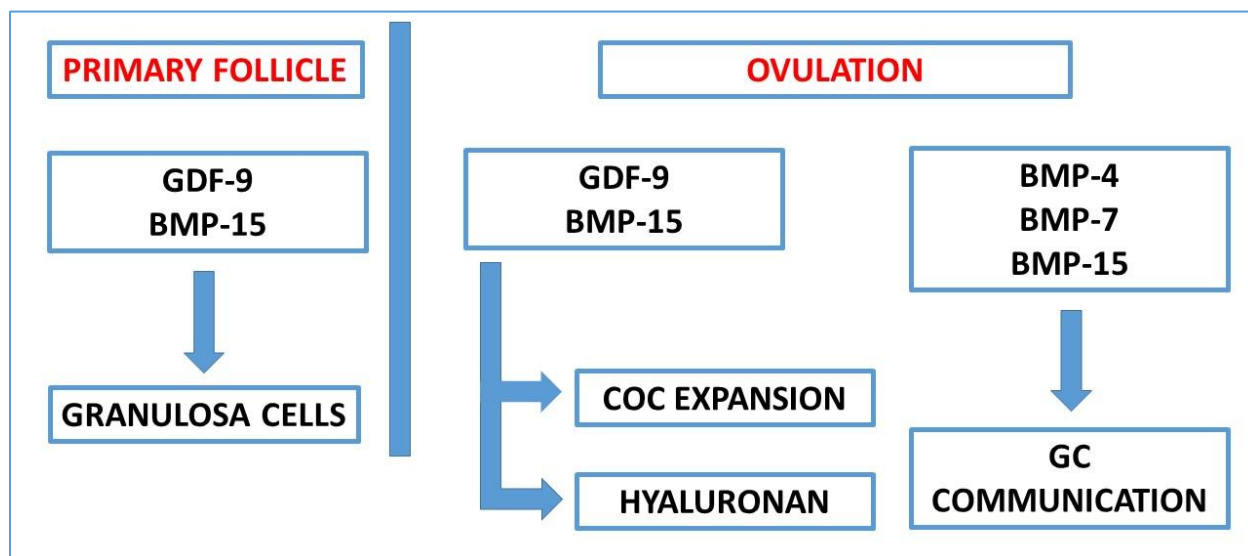


Figure 3. Flow diagram exhibiting the functional roles of GDF-9 and BMP-15 in folliculogenesis and during ovulation. GDF-9 and BMP-15 help in the transition of the primordial follicle to the primary follicle stage and regulate the function of granulosa cells (GC), granulosa cell (GC) communication, cumulus oocyte complex (COC) expansion and hyaluronan production leading to ovulation. Modified from Sanfins et al. (2018)

No work has been published regarding the GDF-9 secretion of the human oocyte after ovulation or even after fertilization. Whether these embryos continue to secrete GDF-9 as it divides and develops into blastocyst is still unknown. There has been evidence indicating a rise on GDF-9 levels in the spent culture medium of water buffalo embryos (Figure 4) from Day 1 to Day 3 with a subsequent decrease from the morula to the blastocyst stage (Liu et al. 2019).

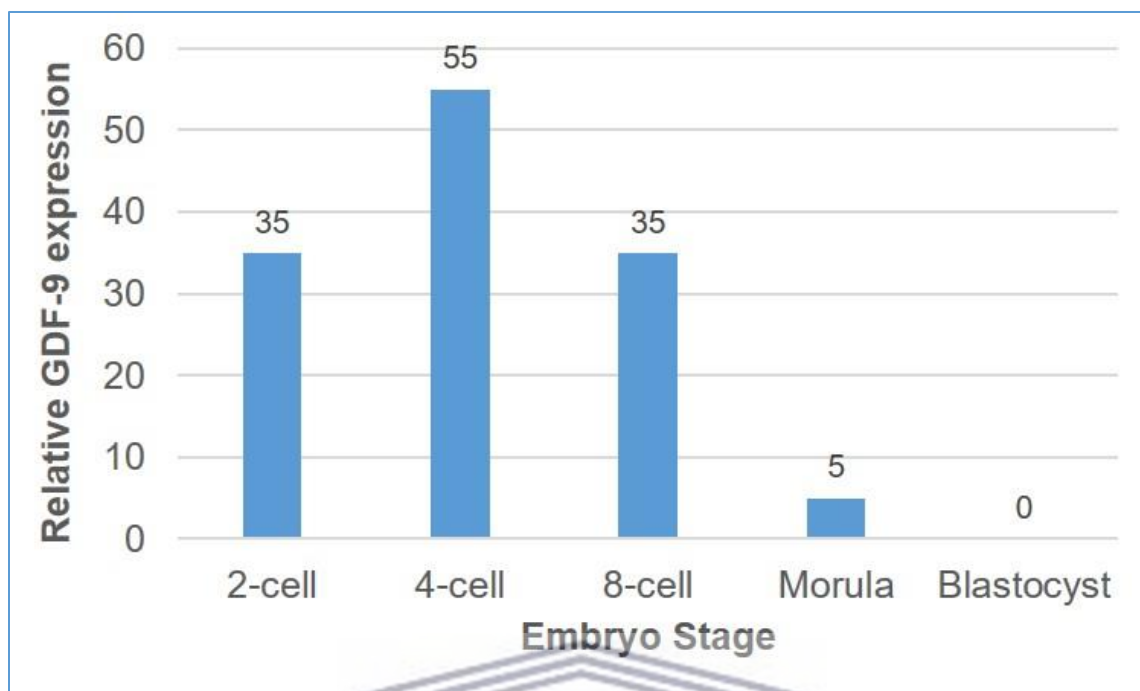
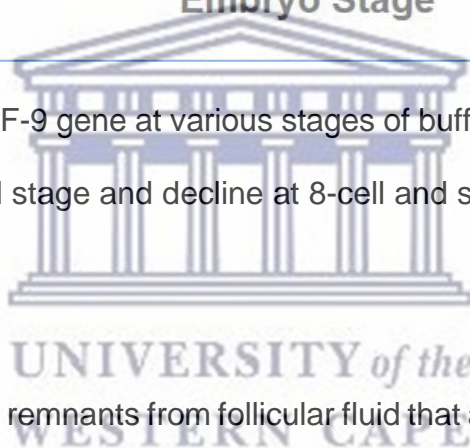


Figure 4. Expression of GDF-9 gene at various stages of buffalo embryogenesis showing an increase up to the 4-cell stage and decline at 8-cell and subsequent stages. Modified from Liu et al. (2019).



High GDF-9 levels could be remnants from follicular fluid that are still present in the oocyte or continued production of GDF-9 from the granulosa cells, which start to decline after Day 3. One possibility is that as the metabolic requirements of the embryo change after Day 3, the secretion of GDF-9 may also change. No work has been published regarding the GDF-9 secretion of the human oocyte after ovulation or fertilization.

Due to the lack of information regarding the presence and function of GDF-9 during embryogenesis, this study aims to detect and measure GDF-9 in spent culture medium and determine if it has any potential as a marker for embryo implantation potential by comparing spent medium of embryos that resulted in a pregnancy and those that did not.

1.7. Oxidation-reduction potential and embryo quality

Oxidative stress, a term that was coined by Sies (1986), occurs when there is an elevation of reactive oxygen species (ROS) that exceeds the level of the body's antioxidant defenses (Agarwal et al. 2003). ROS are oxygen derived molecules such as superoxide anion ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\bullet\text{OH}$) that are produced in low concentrations in the male and female reproductive tracts (Agarwal and Allamaneni 2004). These have the ability to react with any molecule, resulting in structural and functional alterations (Sharma and Agarwal 1996, Agarwal et al. 2006). However, ROS in low concentrations are essential for various bodily functions and processes including sperm physiological processes such as capacitation, hyperactivation, acrosome reaction and sperm-oolemma binding (de Lamirande and Gagnon 1993, Agarwal et al. 2006, de Lamirande and O'Flaherty 2008, Kothari et al. 2010, Guthrie and Welch 2012), but in excess levels can be detrimental to sperm. Excess amounts of ROS are neutralized by various enzymes such as catalase, superoxide dismutase, glutathione peroxidase or reductase, as well as non-enzymatic antioxidants such as vitamin C, vitamin E, vitamin A, pyruvate, glutathione, taurine and hypotaurine (Sikka 2004). Under normal physiological conditions, there is a stable balance between ROS and antioxidants, and a shift towards more ROS will result in oxidative stress (Agarwal et al. 2006).

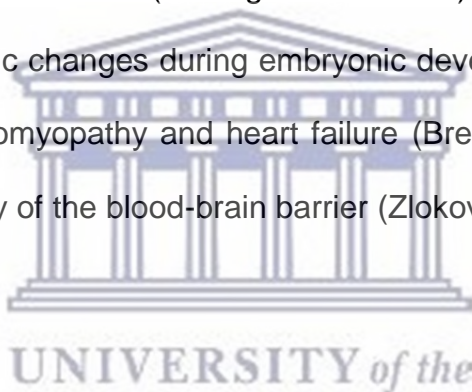
With regard to the role of oxygen, it has been suggested that oxidative stress could affect bovine embryos cultured in atmospheric 20% O_2 due to a higher presence of ROS compared to embryos cultured in 5% O_2 which showed better embryo development and higher blastocyst development rates (Leite et al. 2017). Oxidative stress also affects male

and female embryos differently in that interferon levels in female bovine blastocysts are significantly higher than in males, due to increased activity of the X-linked gene, glucose-6 phosphate dehydrogenase (Kimura et al. 2004, Dennerly 2007).

Oxidative stress has become an important factor that can negatively affect ART outcome (Agarwal et al. 2003, Agarwal and Allamaneni 2004, Pasqualotto et al. 2004, Wiener-Megnazi et al. 2004). Even though ROS have been involved in endometriosis, folliculogenesis, oocyte maturation, hydrosalpingeal fluid, necrozoospermia, asthenozoospermia and sperm DNA damage, these highly reactive molecules have also been implicated in defective embryo development (Goto et al. 1993, Guerin et al. 2001, Agarwal et al. 2006). Oxidative stress has also been shown to negatively affect sperm (Agarwal and Saleh 2002, Sikka 2004). In the female, factors increasing oxidative stress are present in the follicular fluid, such as glutathione peroxidase, which have been shown to correlate positively with pregnancy rates (Paszowski et al. 1995, Attaran et al. 2000), while other markers such as lipid peroxidation, superoxide dismutase activity, and total antioxidant capacity are also associated with fertilization and pregnancy rates after IVF (Sabatini et al. 1999, Oyawoye et al. 2003, Pasqualotto et al. 2004). In contrast, Jozwik et al. (1999) showed that oxidative stress markers such as conjugated dienes, lipid hydroperoxides and thiobarbituric acid-reactive substances in follicular fluid did not reflect the reproductive potential of oocytes.

Oxidative stress reflects an imbalance between oxidants and antioxidants with an overweight of the oxidants. Disturbances in the normal redox (reduction-oxidation) state

of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Further, some reactive oxidative species act as cellular messengers in redox signaling (Schieber and Chandel 2014, Zhang et al. 2016). Oxidative stress can be naturally balanced using antioxidants, such as vitamin C, glutathione, hypotaurine, and taurine, all of which have been shown to protect embryos from excessive ROS (Li et al. 1993). Inadequate resources of antioxidants may lead to increased oxidative stress. Conversely, an excessive amount of antioxidants can lead to reductive stress, which has been shown to be just as harmful as oxidative stress (Castagne et al. 1999). Reductive stress has been shown to induce teratogenic changes during embryonic development (Ufer et al. 2010), cardiac hypertrophy, cardiomyopathy and heart failure (Brewer et al. 2013) as well as decreasing the permeability of the blood-brain barrier (Zlokovic 2011, Mentor and Fisher 2017).



It has been shown that follicular fluid of human oocytes that fertilize have a much higher levels of antioxidants (Oyawoye et al. 2003). However, in the same study, the authors showed that embryos that survived to transfer came from follicles that had significantly lower total antioxidant capacity (TAC), questioning whether TAC is more important in folliculogenesis rather than embryo development. Das et al (2006) showed that there is no significant correlation between ROS levels in follicular fluid and oocyte maturation and quality. However, the authors show significant negative correlation between follicular fluid ROS levels and embryo formation and quality (Das et al. 2006).

Sources of ROS during IVF can be from (1) sperm during vigorous preparation methods, such as centrifugation (Saleh et al. 2003), (2) the removal of antioxidants in the seminal plasma (Agarwal et al. 2006), (3) the metabolism of oocytes since they have been removed from the follicular fluid containing protective antioxidants (Nasr-Esfahani and Johnson 1992, Goto et al. 1993), and (4) external factors such as oxygen environment (Agarwal et al. 2006) since *in vitro* culture is done at higher oxygen concentrations than *in vivo* conditions (Figure 5).

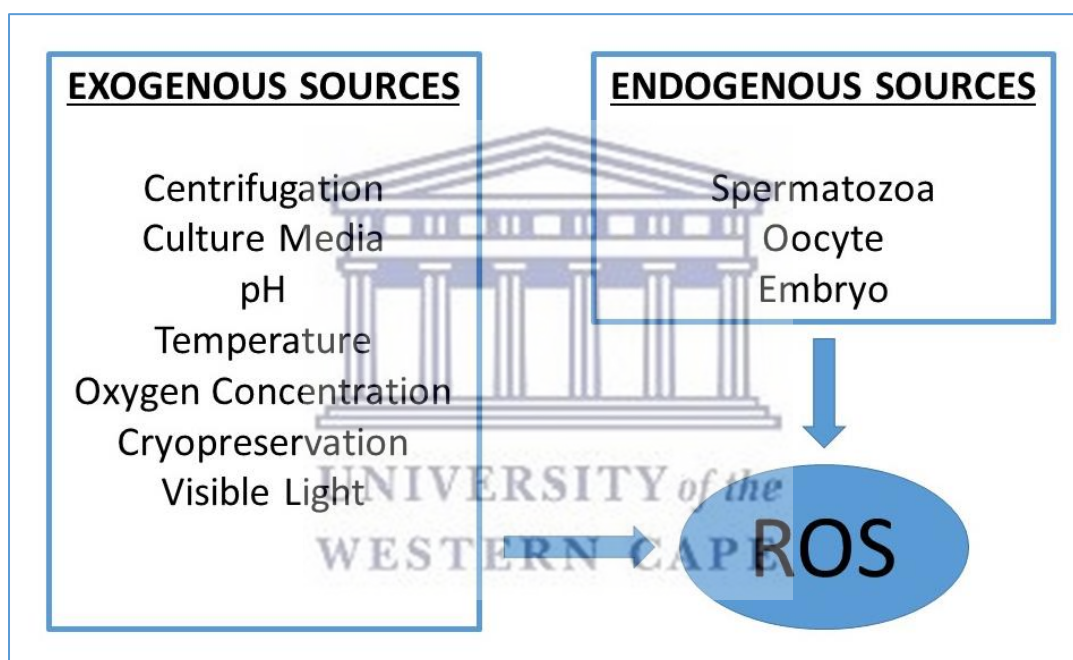
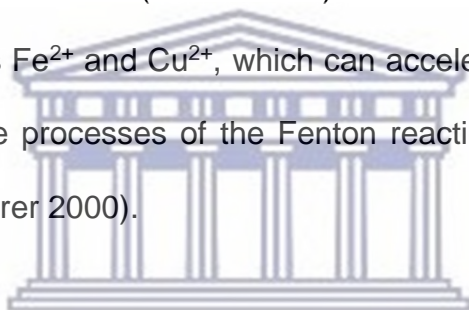


Figure 5. Potential sources of reactive oxygen species. Modified from Agarwal et al. (2014).

Oocytes and embryos produce ROS during energy generation via oxidative phosphorylation (Guerin et al. 2001) and ROS production in embryos is increased *in vitro* (Goto et al. 1993). The oxygen concentration in the fallopian tubes of rabbits and monkeys has been shown to be one-third that of atmospheric oxygen (Mastroianni and Jones 1965,

Maas et al. 1976). Furthermore, the oxygen concentration in the culture medium is 20-fold higher compared to intracellular oxygen (Agarwal et al. 2006) and this may contribute to increased ROS. Culture at 5% O₂ has been shown to decrease the H₂O₂ concentration and improved embryo development in mice (Kwon et al. 1999). Kitagawa et al (2004) have also shown that by reducing the oxygen levels in the incubator from atmospheric 20% to 5% O₂, there was a decrease in H₂O₂ levels, reduction in DNA fragmentation and improvement in embryo development in pigs. Another environmental contributor to ROS production is visible light, as it can lead to photo-oxidative damage of unsaturated lipids and sterols within the membranes (Girotti 2001). Commercial culture media contain various chemicals, such as Fe²⁺ and Cu²⁺, which can accelerate the production of ROS (Guerin et al. 2001) by the processes of the Fenton reaction (Winterbourn 1995) and Haber-Weiss reaction (Kehrer 2000).



There are several interventions, which can help reduce oxidative stress (Figure 6). Supplements to culture media such as serum albumin have been shown to have antioxidant properties (Alvarez and Storey 1983) and can therefore help balance oxidative stress. Others have shown that additives such as glutathione, glutamine or hypotaurine may also help level out the redox status and improved blastocyst development (Ozawa et al. 2006, Suzuki et al. 2007).

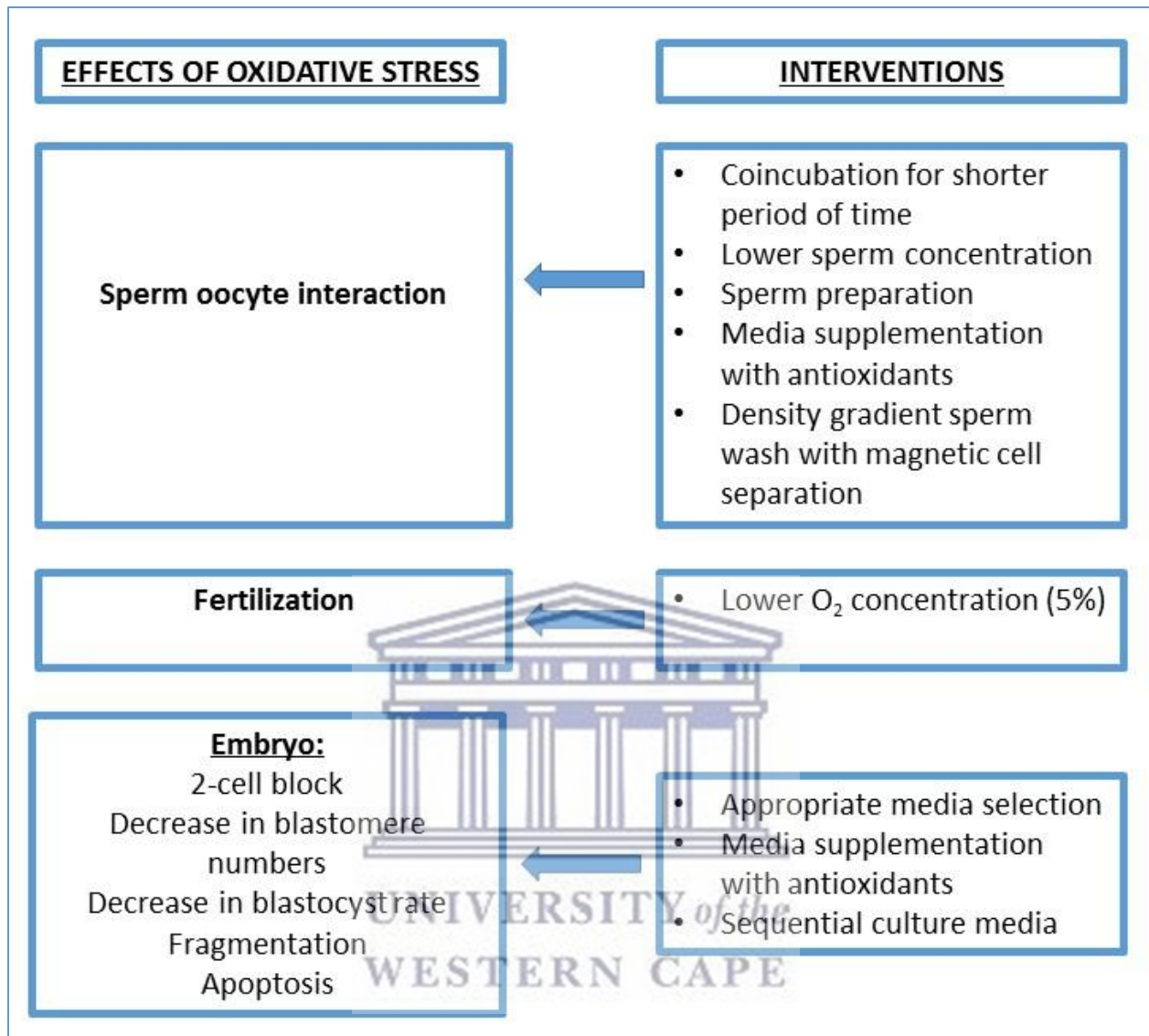


Figure 6. Interventions that can be used to reduce the effects of oxidative stress at different stages of ART. Modified from Agarwal et al. (2014).

Furthermore, IVF may result in increased ROS originating from the oocytes, cumulus cells and sperm (Goto et al. 1993), especially since these are in culture about 14-18 hr before removal of the fertilized oocytes into new culture media. Prolonged exposure of oocytes to sperm during conventional insemination can increase the damage caused by ROS (Aitken et al. 1991, Nallella et al. 2005). Some authors have reported beneficial effects of

shortening this incubation time (Gianaroli et al. 1996, Quinn et al. 1998, Kattera and Chen 2003), while others have shown the opposite (Boone and Johnson 2001, Gil et al. 2004).

In ICSI, however, cumulus cells are stripped from the oocyte and only one sperm is injected into the oocyte, thus eliminating the prolonged exposure to cumulus cells and sperm, which could potentially produce ROS. Interestingly though, the culture medium for embryos created via IVF versus ICSI showed no significant differences in ROS (Bedaiwy et al. 2004). ROS have also been implicated in embryo fragmentation (Yang et al. 1998), showing that fragmented embryos have a significantly higher level of H₂O₂ and also apoptosis compared to non-fragmented embryos. The developing embryo has high energy demands, generating ATP through oxidative phosphorylation and glycolysis (Thompson et al. 2000) which can result in an increase in ROS production.

ROS and total antioxidant capacity (TAC) in the culture medium on Day 1 have been shown to correlate with embryo development and clinical pregnancy (Bedaiwy et al. 2004, Bedaiwy et al. 2006), making them potential predictive metabolic markers. The more rapidly an embryo develops, the more ROS will be generated due to the embryos' metabolic processes (Bedaiwy et al. 2004). In this context, high ROS levels have been associated with poor embryo development and embryo fragmentation (Bedaiwy et al. 2010). Since improved media now allow for the culture of embryos to the blastocyst stage, Bedaiwy et al (2010) analyzed the culture medium on Day 3. They found that Day 3 culture medium from embryos that resulted in a pregnancy showed significant lower ROS levels than medium with embryos that did not yield a pregnancy. This finding was evident

in both IVF as well as ICSI cases, and the authors concluded that increasing levels of ROS in Day 3 culture medium may have a detrimental effect on embryo development. However, the authors did not analyze the culture medium on day 5 (at the blastocyst stage), nor did they use the Day 3 ROS levels to predict blastocyst development rate. They did hypothesize that the relationship between embryo quality and high Day 3 ROS levels is one of cause-and-effect in that high ROS levels may compromise embryo quality through inducing irreversible oxidative stress damage (Bedaiwy et al. 2010).

Since it is clear that both oxidative stress (excess oxidants) and reductive stress (excess antioxidants) are both harmful (Castagne et al. 1999), it is becoming more important to evaluate this balance in order to get a better understanding of the physiological environment and how it impacts sperm, oocytes and embryos. Traditional assays to assess oxidative stress include (1) chemiluminescence to measure ROS (Aitken et al. 1992, Agarwal et al. 2004, Agarwal et al. 2015), (2) calorimetric assays to measure antioxidants (Miller et al. 1993, Mahfouz et al. 2009, Kumar et al. 2014, Marshburn et al. 2014, Kasperczyk et al. 2015, Roychoudhury et al. 2016), (3) thiobarbituric acid assay or 4-hydroxynonenal (Grotto et al. 2007, Weber et al. 2013), (4) apoptotic markers such as annexin V (Moustafa et al. 2004, Tremellen and Tunc 2010), and (5) proteomic tools to measure oxidative stress modified protein alterations (Hamada et al. 2013, Sharma et al. 2013). There are several direct and indirect assays which can measure oxidative stress (Sharma and Agarwal 1996). ROS can be measured using chemiluminescence whereas TAC can be measured using a calorimetric assay, and these results are used to calculate a ROS-TAC score (Sharma et al. 1999, Mahfouz et al. 2009, Agarwal et al. 2015).

However, the limitations of these tests are that they are time-consuming, cumbersome, requiring sophisticated instrumentation and large sample volumes.

Oxidation-reduction potential (ORP) is a relatively new parameter and is a measure of the balance between total oxidants (i.e., oxidized thiols, superoxide radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide, peroxynitrite, transition metal ions, etc.) and total reductants (i.e., free thiols, ascorbate, α -tocopherol, β -carotene, uric acid, etc.) in a sample. Antioxidant capacity reserve (AOC) is a result of exhausting all the antioxidant species, which gives a measure of antioxidants available to counterbalance the oxidative stress. In effect, ORP levels give a snapshot of the redox balance in any given sample (Panner Selvam et al. 2018). ORP correlates well with illness and severity of injury in trauma patients (Shapiro 1972, Rael et al. 2007, Rael et al. 2009, Rael et al. 2009). Semen samples with significantly higher ORP values than control samples expressed more ROS as the redox balance was shifted more to the oxidant state (Agarwal et al. 2006).

A new method for evaluating ORP was introduced (Agarwal et al. 2016) using the MiOXSYS® system (Aytu Bioscience, Englewood, CO, USA), which utilizes galvanostat-based technology. ORP is determined by measuring the transfer of electrons from the antioxidants to oxidants in millivolts and in doing so provides the integrated measure of the existing balance between total oxidants and antioxidants (Agarwal et al. 2016).

Various studies have looked at oxidative stress in culture media at various stages in embryo development, such as Day 1 (Bedaiwy et al. 2004, Bedaiwy et al. 2006), and Day 3 (Bedaiwy et al. 2010, Panner Selvam et al. 2018) and compared the ORP values of several commonly used three sperm preparation and seven IVF culture media. The sperm preparation media showed significantly higher ORP levels (ranging from 233-269mV) than IVF culture media (ranging from 208-273mV). Sage One-Step™ medium showed the lowest ORP (Panner Selvam et al. 2018), which could explain the increased blastocyst formation and delayed DNA methylation observed in single-step media compared with sequential media (Sfontouris et al. 2016), indicating that the choice of medium may play a role in fertilization and embryo development by its redox potential (Panner Selvam et al. 2018). This is in concordance that the relative production of ROS in the oviduct after fertilization is lowered for embryos to implant (Ufer et al. 2010). Since oxidative stress has been shown to affect embryo development (Harvey et al. 2002, Dennerly 2007), it is important to take the choice of IVF culture medium into consideration.

The culture medium collects all the metabolic by-products of the embryos (Paszkowski and Clarke 1996, Bromer and Seli 2008). The metabolic profile of the spent medium has been shown to be a better predictor of implantation than assessment of embryo morphology (Seli et al. 2007, Scott et al. 2008, Seli et al. 2008, Vergouw et al. 2008, Sallam et al. 2016), with oxidative markers being the most predictive (Seli et al. 2007, Bromer and Seli 2008).

ROS levels in spent medium on Day 1 and Day 3 have already been shown to affect embryo development and clinical pregnancy (Bedaiwy et al. 2004, Bedaiwy et al. 2006, Bedaiwy et al. 2010). However, these studies looked at group culture and could not provide medium from individual embryos or details of the specific embryo used for transfer. Subsequent studies were directed at individual spent medium (Wiener-Megnazi et al. 2011, Alegre et al. 2019). Using a thermochemiluminescence assay, it was shown that spent media from Day 3 embryos that resulted in a pregnancy had a significantly higher oxidative status than media from embryos that did not implant (Wiener-Megnazi et al. 2011). Interestingly, it has also showed for the first time that spent media with embryos had higher oxidative levels than blank controls, suggesting that the embryo does exert an “oxidative load” into the surrounding medium (Wiener-Megnazi et al. 2011).

With the advent of new technologies, studies have incorporated the use of time-lapse morphokinetics in combination with oxidative stress measurements of spent culture medium (Alegre et al. 2019). Spent media on Day 5 of embryos that reached blastocyst stage (either transferred or vitrified) showed significantly higher levels of oxidative stress than media from embryos that did not reach blastocyst stage (Alegre et al. 2019). Furthermore, spent media of embryos that implanted also had significantly higher oxidative parameters than those that did not implant. However, Alegre et al (2019) did not report on specific time points but presented an algorithm that took morphokinetics and oxidative stress into consideration. This was a better predictor of implantation rate than embryo morphology or morphokinetics alone (Alegre et al. 2019). These findings are contradictory to the traditional theory that higher ROS negatively influences embryo

development. The authors hypothesized that high-quality embryos have a more extensive oxidative metabolism as seen by the by-products which are secreted into the surrounding medium. Oxygen consumption has been shown to be higher in embryos that result in pregnancy compared to those that do not (Tejera et al. 2016). The idea that ROS in spent media is a function of embryo metabolism and that high quality embryos contribute to oxidative stress as a product of their metabolism, rather than ROS in the media impairing embryo growth is a relatively new development in oxidative stress studies.

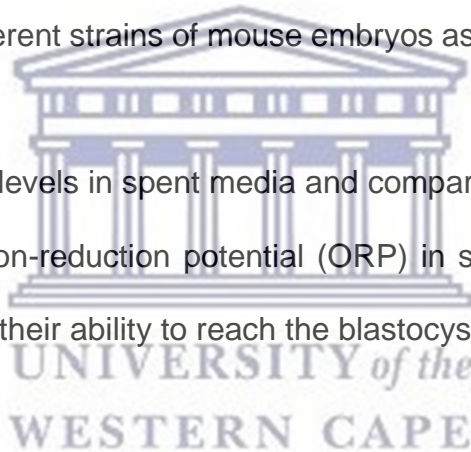
Since there is great discrepancy in the literature, it is important to investigate oxidative parameters in spent media and their role as predictors of embryo health and implantation potential. This study aims to focus on measuring and comparing oxidative reduction potential (ORP) in the spent culture medium of embryos that developed into blastocysts and those that arrested at the cleavage stage, to determine if oxidative stress plays a role in embryo development.



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2. OBJECTIVES

- 2.1. To compare blastocyst development in human embryos cultured in sequential media and signal-step media in two different incubation systems.
- 2.2. To establish if there is a benefit to embryo development by adding IgF-1 or insulin to the culture medium using mouse embryos in term of blastocyst development and time-lapse morphokinetics.
- 2.3. To assess if low oxygen (2%) environment yield better development in mouse embryos with regards to blastocyst development and time-lapse morphokinetics.
- 2.4. To evaluate two different strains of mouse embryos as a better model for research and quality control.
- 2.5. To measure GDF-9 levels in spent media and compare to pregnancy outcome.
- 2.6. To compare oxidation-reduction potential (ORP) in spent media of good quality Day 3 embryos and their ability to reach the blastocyst stage.



3. MATERIALS AND METHODS

3.1. ETHICS AND INSITUTIONAL REVIEW BOARD APPROVAL

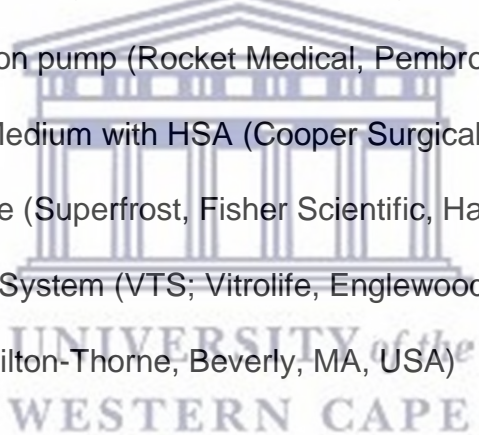
All patient studies were approved by the Institutional Review Board of Baylor College of Medicine under the following protocols: H-39094 (Characterization of clinical and laboratory parameters and their effects on pregnancy outcomes and IVF), H-43271 (Reproductive endocrinology research consortium: In-vitro fertilization (IVF) lab biorepository), and H-44236 (Analysis of spent culture media for biomarkers associated with embryo quality). These studies were also approved by the Ethics Committee of the University of the Western Cape.

3.2. LIST OF MATERIAL AND SUPPLIERS

- 1 mL tuberculin syringe (Fisher Scientific, Hampton, NH, USA)
- 100 mm Falcon® petri dishes (Fisher Scientific, Hampton, NH, USA)
- 135 µm stripper tips (Cooper Surgical, Trumbull, CT, USA)
- 14 mL round bottom Falcon® test tubes (Fisher Scientific, Hampton, NH, USA)
- 18G egg aspiration needle (Cook Medical, Bloomington IN, USA)
- 290 µm stripper pipet tips (Cooper Surgical, Trumbull, CT, USA)
- Biopsy micropipette (Vitrolife, Englewood, CO, USA)
- Cetrorelix acetate (Cetrotide®, Freedom Fertility Pharmacy, Byfield, MA, USA)
- Cryolock straw (Irvine Scientific, Santa Ana, CA, USA)

- DAPI mounting medium (Vector Laboratories, Burlington, CA, USA)
- EmbryoScope™ time-lapse incubator (Vitrolife, Englewood, CO, USA)
- EmbryoSlide™ culture dishes (Vitrolife, Englewood, CO, USA)
- Falcon® 50 mm culture dish (Fisher Scientific, Hampton, NH, USA)
- Falcon® 60 mm organ culture dish (Fisher Scientific, Hampton, NH, USA)
- Falcon® 60mm culture dish (Fisher Scientific, Hampton, NH, USA)
- Follitropin alfa (Gonal F®, MD Serono, Rockland, MA, USA)
- Follitropin beta (Follistim®, Merck and Co., Whitehouse Station, NJ, USA)
- Frozen one-cell mouse embryos (Charles River Laboratories, Wilmington, MA, USA)
- FSH/LH menotropin (Menopur®, Ferring Pharmaceuticals, Parsippany, NJ, USA).
- G100 gas analyzer (ViaSensor, Dexter, MI, USA)
- GDF-9 ELISA kit (Ansh Labs, Webster, TX, USA).
- Global® medium (Cooper Surgical, Trumbull, CT, USA),
- Global® Total medium with HEPES buffer (Cooper Surgical, Trumbull, CT, USA)
- hCG (Novarel®, Ferring Pharmaceuticals, NJ, USA)
- Holding micropipette (Vitrolife, Englewood, CO, USA)
- Hyaluronidase (Cooper Surgical, Trumbull, CT, USA)
- IGF-1 (Millipore Sigma, St. Louis, MO, USA)
- Injection micropipette (Vitrolife, Englewood, CO, USA)
- Insulin (Millipore Sigma, St. Louis, MO, USA)
- Irvine Scientific Vitrification kit (Irvine Scientific, Santa Ana, CA, USA)
- Irvine Scientific Warming Kit (Irvine Scientific, Santa Ana, CA, USA)

- Makler® counting chamber (Cooper Surgical, Trumbull, CT, USA)
- MiOXSYS® system (Aytu Biosciences, Englewood, CO, USA)
- Olympus BX43 fluorescence microscope (Olympus Scientific, Waltham, MA, USA)
- Ovoil™ mineral oil (Ovoil™, Vitrolife, Englewood, CO, USA)
- Polyvinylpyrrolidone (PVP, Cooper Surgical, Trumbull, CT, USA)
- Progesterone gel (Crinone®, Allergan USA, Irvine, CA, USA)
- Quinn's Advantage™ Serum Protein Substitute (SPS; Cooper Surgical, Trumbull, CT, USA)
- Quinn's™ Sperm Washing Medium (Cooper Surgical, Trumbull, CT, USA)
- Rocket Craft™ suction pump (Rocket Medical, Pembroke, MA, USA)
- Sage One-Step™ Medium with HSA (Cooper Surgical, Trumbull, CT, USA)
- Superfrost glass slide (Superfrost, Fisher Scientific, Hampton, NH, USA)
- Viscosity Treatment System (VTS; Vitrolife, Englewood, CO, USA)
- Zilos-tk® laser (Hamilton-Thorne, Beverly, MA, USA)



3.3. MOUSE STUDIES

3.3.1. Mouse strains

Two different strains of mouse embryos were used: (1) B6D2F1 and B6C3F1 hybrid, which is a commonly used model for IVF quality control, and (2) C57BL/6NCrl. The B6D2F1 and B6C3F1 strain is a hybrid of two other hybrids (C57BL/6NCrl & DBA/2NCrl

with (C57BL/6NCrI & C3H/HeNCrI), whereas the C57BL/6NCrI is an inbred strain (Figure 7).

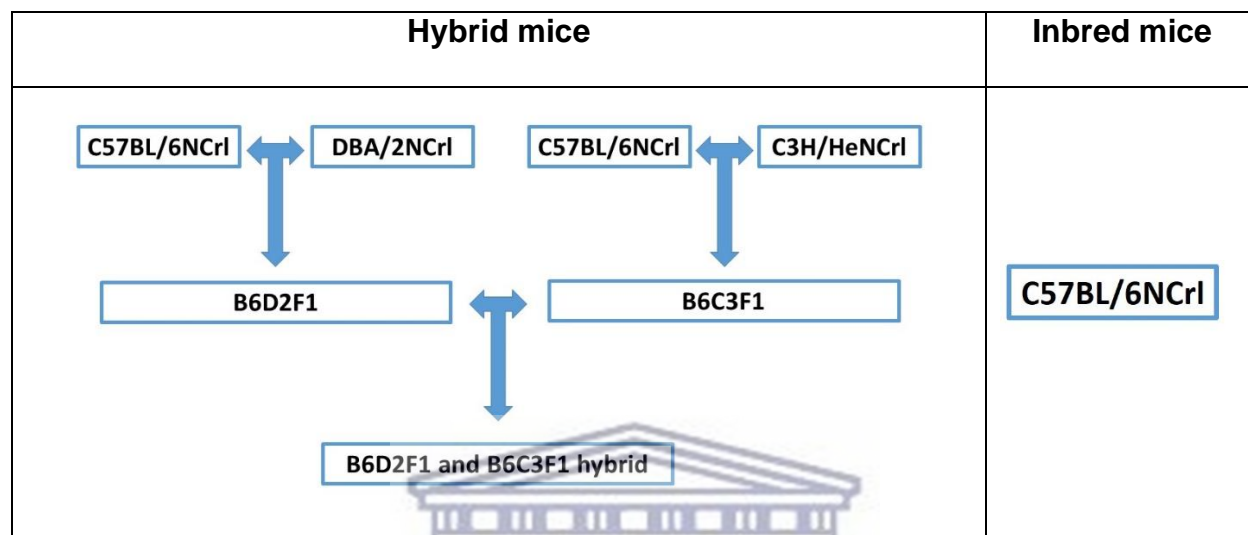


Figure 7. Mouse strains and their lineage

Frozen one-cell mouse embryos were obtained from Charles River Laboratories (Wilmington, MA, USA) and stored in liquid nitrogen dewars until time of thawing.

3.3.2. Thawing of mouse embryos

Commercially obtained frozen one-cell mouse embryos (Charles River Laboratories, Wilmington, MA, USA) were thawed by removing the cryostraw from liquid nitrogen and resting it horizontally on a rack at room temperature for 2 min. The contents of the straw were then mixed by snapping the straw 3-4 times to allow all the medium to mix and be pushed towards the cotton plug. The straw was placed vertically in a water bath at 25°C with the cotton plug down for 1 minute and then with the cotton plug up for another minute.

The straw was removed from the water bath and wiped dry. The lower heat seal was removed by cutting the straw at the midpoint of the air column and the upper heat seal was removed by cutting at the midpoint of the cotton plug. The contents of the straw were expelled in a petri dish using the provided stylet. The embryos were recovered and placed in Global® Total + HEPES medium (Cooper Surgical, Trumbull, CT, USA) at 37°C for 10 min on a stage warmer to allow for rehydration. Embryos were then washed and placed in pre-equilibrated 1 mL Sage One-Step™ medium (Cooper Surgical, Trumbull, CT, USA) in an 60 mm Falcon® organ culture dish (Fisher Scientific, Hampton, NH, USA) before allocating in different test groups.

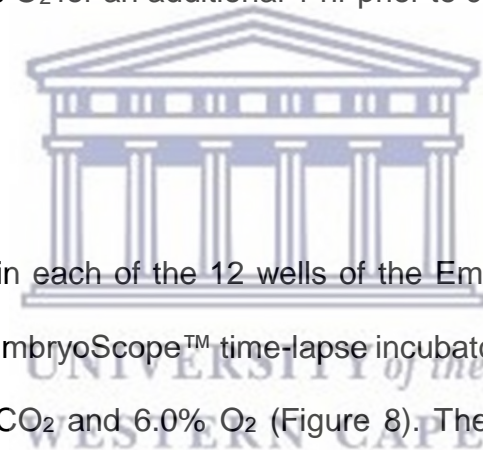
3.3.3. Culture medium and dish preparation

Sage One-Step™ Medium with human serum albumin (HSA) (Cooper Surgical, Trumbull, CT, USA) was used as the control medium for mouse embryo culture. A stock solution of 100 mg/mL insulin (Millipore Sigma, St. Louis, MO, USA) and a stock solution of 100 mg/mL IGF-1 (Millipore Sigma, St. Louis, MO, USA) was prepared and stored at -20°C until use. One-Step™ medium was supplemented with a final concentration of either 100 ng/mL insulin or 100 ng/mL IGF-1. All batches of the culture medium were equilibrated for at least 2 hr in loose capped test tubes in a HeraCell tri-gas incubator (Fisher Scientific, Hampton, NH, USA) set at 37°C, 5.5% CO₂ and 5.0% O₂, prior to preparing EmbryoSlide™ culture dishes (Vitrolife, Englewood, CO, USA). Mineral oil (Ovoil™, Vitrolife, Englewood, CO, USA) was also pre-warmed at 37°C for at least 2 hr prior to dish preparation.

EmbryoSlide™ culture dishes were prepared in an enclosed IVF Chamber (Cooper Surgical, Trumbull, CT, USA) controlled with 6.0% CO₂ at 37°C. Each well in the EmbryoScope™ dish was seeded with a drop of medium using a 135 µm Stripper pipette (Cooper Surgical, Trumbull, CT, USA), and then filled with 25 µL of medium using an Eppendorf pipettor (Fisher Scientific, Hampton, NH, USA). Dishes were prepared with One-Step™ medium, One-Step™ medium with insulin, or One-Step™ medium with IGF-1. The media were overlaid with 1.4 mL of pre-warmed mineral oil and allowed to equilibrate in a Planer B37 benchtop incubator (Cooper Surgical, Trumbull, CT, USA) at 37°C, 5.5% CO₂ and 5.0% O₂ for an additional 1 hr prior to culture.

3.3.4. Embryo culture

One embryo was placed in each of the 12 wells of the EmbryoSlide™ (Figure 8). The slides were placed in the EmbryoScope™ time-lapse incubator (Vitrolife, Englewood, CO, USA) set at 37°C, 5.5% CO₂ and 6.0% O₂ (Figure 8). The camera was set to record images every 10 min for a total of 6 days of culture. Time-lapse videos were annotated for the various cell division stages.



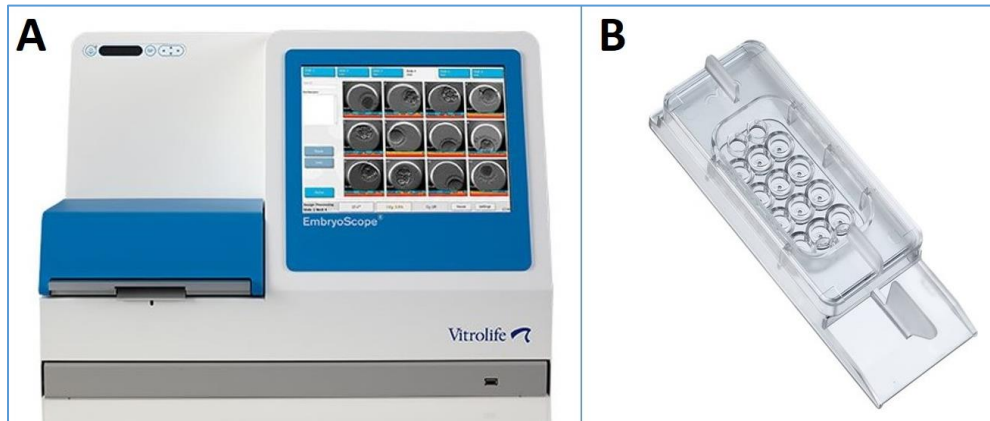


Figure 8. EmbryoScope™ time-lapse incubator (A) with EmbryoSlide™ culture dish with 12 individual culture wells (B). Reprinted with permission from Vitrolife, Englewood, CO, USA (<https://www.vitrolife.com/products/time-lapse-systems/embryoscope-time-lapse-system/>).

3.3.5. Ultra-low (2%) oxygen culture conditions

For the ultra-low oxygen studies, the EmbryoScope™ was set at 37°C, 5.5% CO₂ and 2.0% O₂. The gas concentrations were independently verified using a G100 gas analyzer (ViaSensor, Dexter, MI, USA) prior to culture. Thawed one-cell mouse embryos were cultured in Sage One-Step™ medium for 6 days. The various cell division time points were annotated and compared to those in the 6% O₂ culture condition.

3.3.6. Time-lapse microscopy and morphokinetic annotations

Images of mouse embryo development were recorded every 10 min at 7 different focal planes on the EmbryoScope™, from the start of culture until Day 6 (Figure 9).

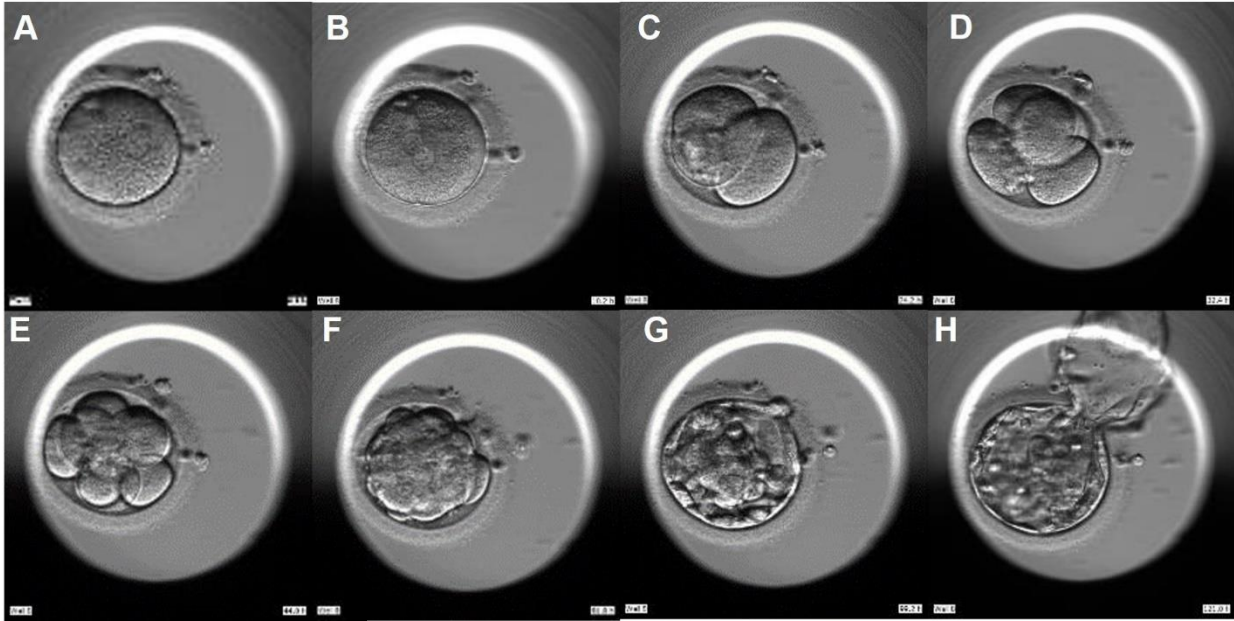


Figure 9. Images captured from EmbryoScope™ showing (A) mature MII oocyte after ICSI, (B) 2PN, (C) 2-cell, (D) 4-cell, (E) 8-cell, (F) morula, (G) blastocyst, and (H) hatching blastocyst.

All morphokinetic parameters were annotated manually using EmbryoViewer software (Figure 10) by the same embryologist to eliminate bias. Time-lapse videos were annotated for various time points associated with cell division as follows: 2-cell (t_2), 3-cell (t_3), 4-cell (t_4), 5-cell (t_5), 6-cell (t_6), 7-cell (t_7), 8-cell (t_8), start of compaction (t_{SC}), morula (t_M), start of blastulation (t_{SB}), blastocyst (t_B), expanded blastocyst (t_{EB}) and hatching blastocyst (t_{HB}). In order to standardize all time points, the time to reach 2-cells was used as the starting point since the exact time of insemination was unknown. All subsequent times were adjusted by subtracting t_2 from each time point making t_2 as 0 hr.

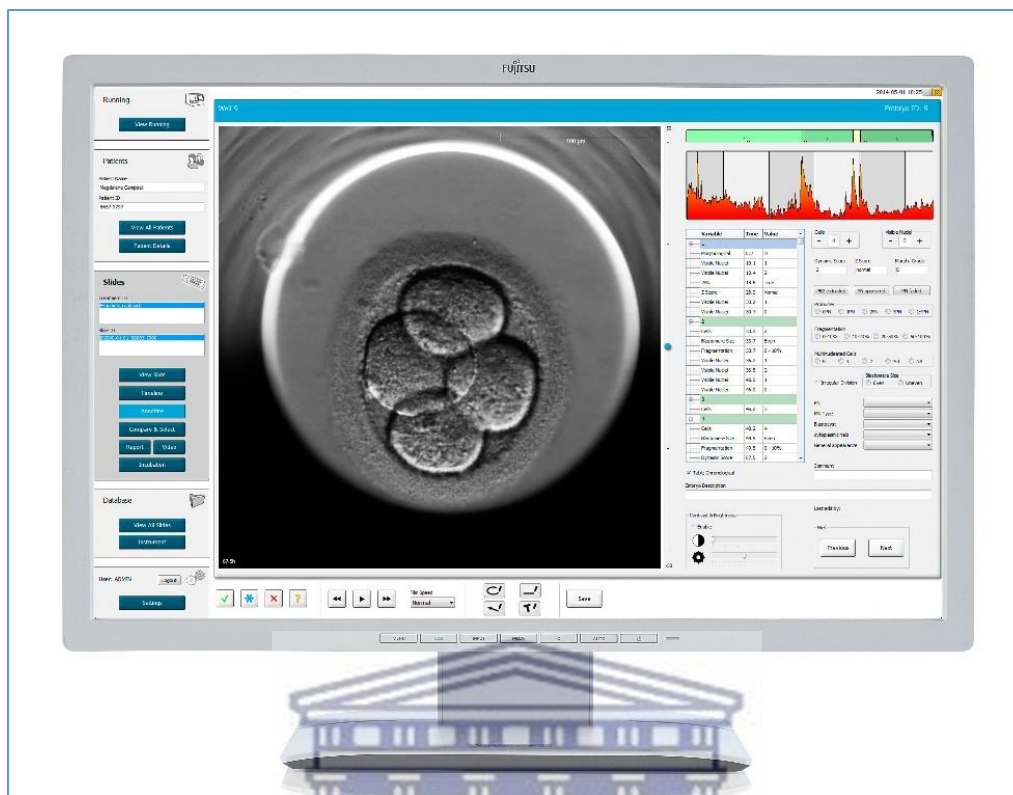


Figure 10. EmbryoViewer™ time-lapse annotation software used to annotate various cell division time points. Reprinted with permission from Vitrolife (Englewood, CO, USA).

3.3.7. Fluorescence imaging of cell counts

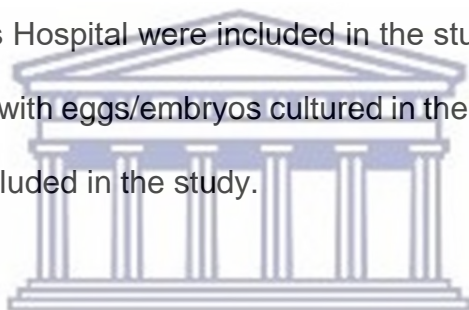
For the low oxygen study, fluorescence imaging was performed on mouse embryos cultured in 6% O₂ and 2% O₂ to determine cell counts. A 3µL drop of Vectashield Mounting Medium with DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories, Burlington, CA, USA) was placed on a glass slide (Superfrost, Fisher Scientific, Hampton, NH, USA). An embryo was added to each DAPI drop and immediately covered with 22x50 mm coverslip (Fisher Scientific, Hampton, NH, USA). The coverslip was gently tapped to flatten the embryos and slides were stored overnight prior to observing.

Embryos were viewed using an Olympus BX43 fluorescence microscope (Olympus Scientific Solutions, Waltham, MA, USA) on the DAPI setting at 460 nm. Stained cells were counted in duplicate for each embryo.

3.4. HUMAN STUDIES

3.4.1. Patients

All patients undergoing infertility treatment with in-vitro fertilization at the Family Fertility Center at Texas Children's Hospital were included in the study, irrespective of their age or diagnosis. Only patients with eggs/embryos cultured in the EmbryoScope™ time-lapse incubation system were included in the study.



3.4.1.1. GDF-9 study

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Only patients meeting the following criteria were included in the study:

- Intracytoplasmic sperm injection (ICSI)
- Pre-implantation genetic testing for aneuploidy (PGT-A)
- Frozen embryos transfers with single euploid embryos
- Day 5 and Day 6 blastocysts (Day 7 excluded)

GDF-9 levels in the spent media, along with time-lapse morphokinetics were compared with pregnancy outcome, which were divided into 4 groups:

- Non-pregnant (negative β -HCG, 2 weeks post ET)

- Biochemical pregnancies (positive β -HCG, 2 weeks post ET)
- Clinical pregnancies (heartbeat 6 weeks post ET)
- Early miscarriage (no heartbeat 6 weeks post ET)

3.4.1.2. Oxidative reduction potential study

Only patients meeting the following criteria were included in the study

- Intracytoplasmic sperm injection (ICSI)
- Good quality Day 3 embryos (8 cell, Grade B)

Spent media were evaluated on Day 6 for good quality embryos that developed to the blastocyst stage and those that did not. The grading system for embryos are as follows: Grade A (symmetrical blastomeres and no fragmentation), Grade B (slightly asymmetrical blastomeres and <15% fragmentation), Grade C (asymmetrical blastomeres and 15-50% fragmentation), Grade D (undistinguishable blastomeres with >50% fragmentation).

3.4.2. Ovarian stimulation

Each patient underwent an individualized antagonist ovarian stimulation protocol using a combination of FSH, either follitropin alfa (Gonal F®, MD Serono, Rockland, MA, USA) or follitropin beta (Follistim®, Merck and Co., Whitehouse Station, NJ, USA), and FSH/LH menotropin (Menopur®, Ferring Pharmaceuticals, Parsippany, NJ, USA). Follicular number and size were obtained via ultrasound during ovarian stimulation monitoring

every two days. Blood serum samples were collected via peripheral venipuncture within one hour of the ultrasound assessment to measure estradiol levels. Once the lead follicle reached 13 mm, cetorelix acetate (Cetrotide[®], Freedom Fertility Pharmacy, Byfield, MA, USA) was administered. When 2 or more follicles measured greater than 18 mm with appropriate estradiol values, ovulation was triggered by administering 10,000 units of hCG (Novarel[®], Ferring Pharmaceuticals, Parsipanny, NJ, USA).

3.4.3. Oocyte retrieval

Thirty-six hours after hCG trigger, transvaginal oocyte aspiration was performed under total intravenous sedation. Follicular fluid was aspirated from each of the follicles via transvaginal ultrasound using an 18G egg aspiration needle (Cook Medical, Bloomington IN, USA) in conjunction with a Rocket Craft[™] suction pump (Rocket Medical, Pembroke, MA, USA) into 14 mL round bottom Falcon[®] test tubes (Fisher Scientific, Hampton, NH, USA). The follicular aspirates were poured into 100 mm Falcon[®] petri dishes (Fisher Scientific, Hampton, NH, USA) and examined for the presence of cumulus oocyte complexes (COC). COC's were collected and rinsed through two 60 mm Falcon[®] culture dishes (Fisher Scientific, Hampton, NH, USA) containing 7.5 mL Global[®] medium supplemented with 12% Serum Protein Substitute (SPS; Cooper Surgical, Trumbull, CT, USA) under oil. All manipulations were done in a humidified IVF Chamber (Cooper Surgical, Trumbull, CT, USA) set at 37°C and 6% CO₂, and these settings were verified independently every morning before the procedure. Any debris or blood clots adhering the cumulus was dissected using two syringe needles (Fisher Scientific, Hampton, NH, USA). After the procedure, all oocytes were placed in a Falcon[®] 60 mm organ culture

dish (Fisher Scientific, Hampton, NH, USA) containing Global® medium with 12% SPS (Cooper Surgical, Trumbull, CT, USA) under oil and placed in a Planer B37 bench-top incubator at 37°C, 5.5% CO₂ and 5.0% O₂ for 2 hr prior to sperm injection.

3.4.4. Semen collection and preparation

Male partners were instructed to have 2-3 days of sexual abstinence prior to the procedure. Semen samples were collected via masturbation into a labelled sterile container at the same time as their partner's oocyte retrieval. Patients were asked about their recent medical history, any medications or conditions, days of abstinence, and time of collection. Samples were placed in an incubator at 37°C for 30 min to allow liquefaction to occur. If no liquefaction was noted after 30 min or if the samples exhibited elevated viscosity, Viscosity Treatment System (VTS; Vitrolife, Englewood, CO, USA) was used prior to analysis. A complete vial of VTS was directly added to the sample in the specimen cup, gently mixed and placed in a 37°C incubator for 5 minutes or until liquefied.

Semen analysis was performed for the following parameters: volume, color, pH, sperm concentration, percentage motility, forward progression and presence of round cells and debris. No sperm morphology evaluation was performed on the day of procedure since this would have been done on prior semen analyses. Sperm concentration and motility were determined manually using a Makler® counting chamber (Cooper Surgical, Trumbull, CT, USA). Five microliters of liquefied semen were placed on the chamber and covered with the coverslip. Three regions of 10 squares were counted for motile and immotile sperm and averaged to determine the concentration in millions/mL (motile plus


immotile), and the percentage motility (number of motile sperm divided by total sperm). Total sperm concentration was calculated by multiplying the sperm concentration by the total volume.

The semen sample was placed in a 15 mL Falcon® conical test tube (Fisher Scientific, Hampton, NH, USA) and overlaid with Sperm Washing Medium containing 10% HSA (Cooper Surgical, Trumbull, CT, USA). The sperm were allowed to swim up for at least 1 hr prior to injection.

3.4.5. Intracytoplasmic sperm injection (ICSI)

Prior to ICSI, oocytes were denuded of all cumulus cells by placing them in a 1 mL drop of hyaluronidase (Hyase, Cooper Surgical, Trumbull, CT, USA) for no more than 1 minute. Only 12 oocyte complexes or less were denuded at one time. Oocytes were rinsed in hyaluronidase initially using a Pasteur pipette to remove excess cumulus cells, and then pipetting with 290 µm and 135 µm Stripper pipet tips (Cooper Surgical, Trumbull, CT, USA). After 1 minute, oocytes were placed back into the organ culture dish containing Global® medium with 12% SPS (Cooper Surgical, Trumbull, CT, USA), and rinsed again to remove any further cells that may be attached to the zona pellucida. Oocytes were graded for maturity as (1) metaphase-II by the presence of a polar body, (2) metaphase-I by the absence of both a polar body and germinal vesicle, and (3) prophase-I by the presence of a germinal vesicle. Only metaphase-II oocytes were injected.

An ICSI dish was prepared by placing 7 μ L of pre-warmed (37°C) polyvinylpyrrolidone (PVP, Cooper Surgical, Trumbull, CT, USA) in the center of a Falcon® 50 mm culture dish (Fisher Scientific, Hampton, NH, USA) and surrounded by 4 drops of 7 μ L of pre-warmed (37°C) Global® Total medium with HEPES buffer (Cooper Surgical, Trumbull, CT, USA) at the 12, 3, 6 and 9 o'clock positions. These drops were covered with 4 mL of oil pre-warmed at 37°C (Ovoil™, Vitrolife, Englewood, CO, USA). Using a 135 μ m Stripper tip, a small amount of sperm was aspirated from the top of the media containing the swim-up portion of the sperm and placed in the PVP drop. Oocytes to be injected were placed in the outer drops.



Motile sperm in the PVP drop were immobilized by crushing the tail using the injection micropipette (Vitrolife, Englewood, CO, USA) and aspirated into the pipette tail-first. Using a holding micropipette (Vitrolife, Englewood, CO, USA), the oocyte was held in place with the polar body at either the 6 o'clock or 12 o'clock position to avoid accidentally damaging the meiotic spindle, which has been shown to lie under or close to the polar body. The injection pipette was used to break the oolemma by gentle aspiration, after which the sperm was injected head first into the ooplasm, and the pipette gently removed. The egg was released from the holding pipette and the remaining eggs were injected.

Once all the eggs were injected, they were rinsed through 5 drops of One-Step™ medium under oil and placed in individual wells of the EmbryoSlide™. The slides were then loaded into the EmbryoScope™ incubator set at 37°C, 5.5% CO₂ and 5.0% O₂. The patient

information and time of insemination was entered to set the start point for the time-lapse imaging.

3.4.6. Assisted hatching

For patients requesting preimplantation genetic screening or diagnosis, assisted hatching was performed on all cleaving embryos on Day 3 using a Zilos-tk® laser (Hamilton-Thorne Beverly, MA, USA). Several laser pulses were directed onto the zona pellucida without touching any of the blastomeres. This breach in the zona allows for easy herniating of the blastocyst on day 5 and facilitates trophectoderm biopsy. No media change was done on Day 3.



3.4.7. Trophectoderm biopsy

Once the embryo started herniating on day 5, 6 or 7, the embryos were placed in individually labelled drops of 20 µl Global® Total media with HEPES buffer (Cooper Surgical, Trumbull, CT, USA) covered with mineral oil at 37°C. Embryos were biopsied by holding the embryo closest to the inner cell mass with a holding micropipette (Vitrolife, Englewood, CO, USA). The trophectoderm was aspirated into a biopsy micropipette (Vitrolife, Englewood, CO, USA) by either gentle suction using either mouth pipette or microinjector. Once 4-5 cells were aspirated, the biopsy was separated from the embryo using a combination of laser pulses and gently pulling away from the embryo. After separation, the biopsy was expelled from the micropipette and the embryo was released from the holding micropipette.

The embryo was rinsed and placed in individually labelled drops containing One-Step™ media, until time of vitrification. The biopsy remaining in the drop was aspirated with a 135 µm Stripper tip and rinsed through 3 drops of biopsy buffer supplied by the PGS lab (Igenomix, Cooper, Natera) and placed in 1.5 µL of biopsy buffer into correspondingly labeled PCR Eppendorf tubes. Once all the biopsies were transferred, the PCR tubes were frozen at -20°C until time of shipment to the PCR lab for analysis.

3.4.8. Embryo vitrification

Embryos were cryopreserved via vitrification using Irvine Scientific vitrification kit (Irvine Scientific, Santa Ana, CA, USA). Embryos that were not biopsied were first shrunken by aiming a laser pulse onto the thinnest part of the trophectoderm in order to allow the blastocoel fluid to escape and letting the embryo collapse. Embryos that were biopsied were already shrunken due to the biopsy process.

Each embryo was placed into a correspondingly labelled 40 µL drop of equilibration solution (ES) for at least 2 min at room temperature. Thereafter, the embryo was rinsed through 3 drops of 80 µL vitrification solution (VS) also at room temperature and placed onto the tip of a pre-labelled cryolock straw (Irvine Scientific, Santa Ana, CA, USA) and plunged directly into a liquid nitrogen bath. The exposure time to vitrification solution prior to plunging into liquid nitrogen was between 20 to 40 seconds.

3.4.9. PGS analysis

The PGS analysis was done at one of the following laboratories: Igenomix (Torrence, CA, USA), CooperGenomics (Livingston, NJ, USA) and Natera, Inc (San Carlos, CA, USA). The PGS platform used was next generation sequencing (NGS). Data reported included euploidy status, gender, presence of mosaicism and also mitoscore, which measures mitochondrial integrity (for Igenomix cases).

3.4.10. Embryo warming

Once the PGS data were reviewed with the patient, an embryo was chosen for frozen embryo transfer. On the morning of the transfer, the selected embryo was warmed several hours before the procedure using a Irvine Scientific Warming Kit (Irvine Scientific, Santa Ana, CA, USA) containing WS1 (warmed to 37°C for at least 30 min), and WS2 and WS3 (warmed to room temperature for at least 30 min). These solutions contained decreasing amounts of sucrose (WS1: 1M sucrose; WS2: 0.5M sucrose and WS3: no sucrose).

The cryolock containing the embryo was removed from liquid nitrogen and swiftly plunged into a 1mL drop of pre-warmed WS1 in the center well of organ culture dish, allowing the embryo to float off. After 1 minute, the embryo was aspirated and placed in 100 µL WS2 for 3 min and then into two 100 µL drops of WS 3, first for 3 min and then 1 minute. The embryo was rinsed in 4 drops of Global® medium supplemented with 20% SPS (Cooper Surgical, Trumbull, CT, USA) under oil before placing in the final drop also containing Global® media supplemented with 20% SPS for culture. Assisted hatching was

performed on the embryos using several laser pulses directed onto the zona pellucida. The embryo was placed in a Planer BT37 benchtop incubator set at 37°C, 5.5% CO₂ and 5.0% O₂ until time of transfer. After about 1 hr after warming, the embryo was evaluated for re-expansion of the blastocoel cavity to ensure proper survival after warming.

3.4.11. Embryo transfer

All embryo transfers were performed under ultrasound guidance using one of the following catheters: Guardia™ Access Embryo Transfer Catheter (Cook Incorporated, Bloomington, IN, USA), Echotip® Soft-Pass™ Embryo Transfer Catheter (Cook Incorporated, Bloomington, IN, USA), Sure-Pro Ultra® Embryo Replacement Catheter with Obturator (Copper Surgical, Trumbull, CT, USA). Once all proper patient and embryo identifications were confirmed, the embryo was removed from culture dish and placed in a 100 µL drop of Global® Medium with 20% SPS (Cooper Surgical, Trumbull, CT, USA) without oil, taking care to first rinse the pipette of any oil. A 1 mL tuberculin syringe (Fisher Scientific, Hampton, NH) attached to the embryo transfer catheter was rinsed with Global® Medium with 20% SPS (Cooper Surgical, Trumbull, CT, USA) and flushed up to the 0.01 mL mark. The embryo was aspirated into the catheter with as little medium as possible followed by a column of air.

Once the cervix was rinsed and the outer sheath of the catheter was in place, the inner catheter containing the embryo(s) was placed 1cm from the fundus and the contents of the catheter was expelled. The catheter was removed while keeping the plunger depressed to avoid re-aspirating the embryo. The catheter was flushed with media and

checked for the presence of any retained embryos. In the event of any retained embryos, the catheter was reloaded and the transfer procedure was repeated.

3.4.12. Pregnancy outcome

Luteal support was provided by vaginal progesterone gel (Crinone®, Allergan USA, Irvine, CA, USA). Pregnancy outcome was quantitatively determined 14 days post embryo transfer by measuring serum β -hCG levels. Patients with a positive pregnancy were scanned by ultrasound 6-7 weeks after embryo transfer to determine viability. Those with gestational sacs and fetal heartbeat were considered as clinical pregnancy and those that did not were considered as biochemical pregnancies.

3.4.13. Spent media collection, labelling and storage

Once embryos were removed from the well in the EmbryoScope™ slide, 22 μ L of spent media were aspirated from the well using Eppendorf PCR clean pipette tips (Fisher Scientific, Hampton, NH) taking care not to aspirate any oil. Spent media were placed into pre-labelled low protein 0.2ml BioDot Pure PCR tubes (Dot Scientific Inc., Burton, MI, USA). A new pipette tip was used for each well to avoid contamination media. Extra care was taken to avoid aspirating oil into the tip. For each day of spent media collection, 22 μ L of control media was also aspirated from the rinse wells in the EmbryoScope™ slide and placed into a PCR tube.

The PCR tubes containing spent media were labelled with a de-identified coded patient identification number followed by the embryo number. Control tubes were labelled with the patient identification code, slide number and rinse well number. The slide was placed back into the EmbryoScope™ for further culture of remaining embryos. Spent media tubes were stored in PCR tube racks at -80°C until analysis. A log was kept with patient identification code, tube number, patient name and embryo number.

3.4.14. Time-lapse morphokinetic annotations

All time-lapse morphokinetic parameters starting from ICSI to blastocyst formation were annotated manually using EmbryoViewer software (Figure 10) with t0 being the time of ICSI. The morphokinetic parameters were determined by a team of trained personnel. To decrease annotator bias, morphokinetic annotation, measurement consistency, and internal quality control was verified by the senior embryologist. Parameter information collected included: appearance of 2 pronuclei (tPNa), fading of 2 pronuclei (tPNf), time to 2-cell (t2), t3, t4, t5, t8, t9, time to morula (tM - defined as 50% cells with indistinct membranes), time to start of early blastulation (tSB – defined as <50% blastocoel cavity), time to blastocyst (tB – defined as >50% blastocoel cavity) and time to expanded blast (tEB- 100% blastocoel cavity and thinning of zona pellucida). In cases where a certain cell division was not noted, no value was entered for that time point. Embryos that did not develop to the blastocyst stage were excluded from the study.

3.4.15. Growth differentiation factor 9 (GDF-9) measurements

GDF-9 in spent medium was measured using the AL-176 GDF-9 ELISA kit (Ansh Labs, Webster, TX). All assays were run at the Ansh Labs facilities according to their recommended manufacturer protocols (<https://www.anshlabs.com/wp-content/uploads/inserts/AL176.pdf>)

The GDF-9 ELISA is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls and Unknown samples were added to GDF-9 antibody coated microtiter wells and incubated. After the first incubation and washing, the wells were incubated with biotinylated GDF-9 antibody. After the second incubation and washing, the wells were incubated with streptavidin horseradish peroxidase conjugate (SHRP). After the third incubation and washing step, the wells were incubated with substrate solution (TMB). After TMB incubation, an acidic stopping solution is added.

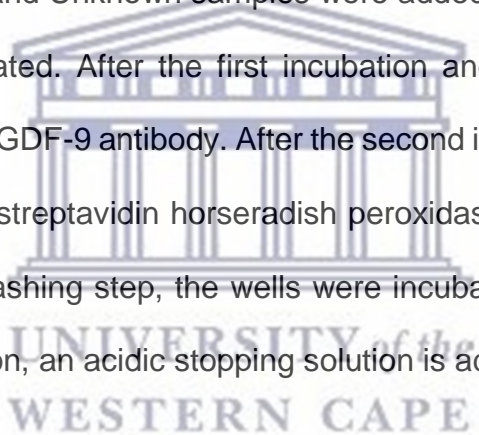




Figure 11. The GDF-9 ELISA kit with all reagents (Reprinted with permission from Ansh Labs, Webster, TX, USA).

In principle, the antibody-biotin conjugate binds to the solid phase antibody-antigen complex, which in turn binds to the streptavidin-enzyme conjugate. The antibody-antigen-biotin conjugate-SHRP complex bound to the well is detected by enzyme-substrate reaction. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as reference filter. The absorbance measured is directly proportional to the concentration of GDF-9 in the samples and calibrators.

3.4.15.1. Preparation of reagents

GDF-9 Calibrator B-F and GDF-9 Controls I & II were each reconstituted, solubilized and mixed with 1 mL deionized water. The Calibrator B/2 solution was prepared using 150 μ L of CAL-B with 150 μ L of Calibrator A/sample Diluent. The wash concentrate was diluted 25-fold with deionized water to prepare the wash solution. The GDF-9 Antibody-Biotin Conjugate Concentrate was diluted at a ratio of 1-part conjugate to 50 parts of BMP-15/GDF-9 Conjugate Diluent, according to the number of wells used.

3.4.15.2. Assay procedure

All specimens and reagents were allowed to reach room temperature ($23 \pm 2^\circ\text{C}$) and mixed thoroughly by gentle inversion before use. Calibrators, controls, and unknowns were assayed in duplicate.



GDF-9 Calibrators B-F and GDF-9 Controls I & II were each reconstituted with 1 mL deionized water and solubilized for 10 min. Fifty μ L of the Calibrators B/2, B-F, Controls and Unknowns were pipetted into the appropriate wells. Fifty μ L of the BMP-15/GDF-9 Assay Buffer were added to each well using a repeater pipette. The plate was incubated shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 3 hr at room temperature ($23 \pm 2^\circ\text{C}$).

During the last 20-30 min of incubation, the GDF-9 Antibody-Biotin Conjugate Solution was prepared by diluting the GDF-9 Biotin Conjugate Concentrate in BMP-15/GDF-9 Conjugate Diluent as described under the Preparation of the Reagents section.

Each strip was aspirated and washed 5 times with Washing Solution (350 μ L/per well) using an automatic microplate washer. One hundred μ L of the Antibody-Biotin Conjugate Solution were added to each well using a repeater pipette. The plate was incubated, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 1 hour at room temperature ($23 \pm 2^\circ\text{C}$). Each strip was again aspirated and washed 5 times with the Wash Solution (350 μ L/per well) using an automatic microplate washer.

One hundred μ L of the Streptavidin-Enzyme Conjugate-RTU were added to each well using a repeater pipette. The plate was incubated, shaking at a fast speed (600-800 rpm) on an orbital microplate shaker, for 30 min at room temperature ($23 \pm 2^\circ\text{C}$). Each strip was again aspirated and washed 5 times with the Wash Solution (350 μ L/per well) using an automatic microplate washer.

One hundred μ L of the TMB chromogen solution were added to each well using a precision pipette, taking care to avoid exposure to direct sunlight. The wells were incubated, shaking at 600–800 rpm on an orbital microplate shaker, for 15-18 min at room temperature ($23 \pm 2^\circ\text{C}$), noting the color development to optimize the incubation time.

One hundred μL of the stopping solution were added to each well using a precision pipette. The absorbance of the solution in the wells was read within 20 min, using a microplate reader set to 450 nm.

3.4.15.3. Determination of results

The mean optical density (OD) for each calibrator, Control, or Unknown was calculated. The log of the mean OD readings for each of the Calibrators were plotted along the y-axis versus log of the GDF-9 concentrations in pg/mL along the x-axis, using a cubic regression curve fit. The GDF-9 concentrations of the Controls and unknowns were determined from the calibration curve by matching their mean OD readings with the corresponding GDF-9 concentrations. Any sample reading higher than the highest Calibrator was appropriately diluted with the 0 pg/mL (CAL A) and re-assayed. Any sample reading lower than the analytical sensitivity was reported as such.

3.4.16. Oxidation-reduction potential (ORP) measurements

ORP was measured using the MiOXSYS® system (Aytu Biosciences, Englewood, CO, USA) according to manufacturer's guidelines that accompanied the unit. The MiOXSYS® machine was calibrated before any tests were run to ensure they met the calibration standards by using preset standard chips. The readings were recorded and confirmed that they were within the calibration range.

3.4.16.1. Principle of the Procedure

The MiOXSYS® System (Figure 12) is based on electrochemical technology, which uses a platinum-based electrode sensor with an Ag/AgCl reference cell, and a galvanostat-based analyzer, which completes the circuit. Once the sample is placed on the sensor and placed into the analyzer, the sample is allowed to flow across the working electrode and to fill the reference cell, thereby completing the electrochemical circuit. Once the electrode surfaces detect the sample, the voltage is measured between the reference cell and working electrode every 0.5 seconds (or 2 Hz), while the counter is set to a voltage sufficient to achieve a 1 nA oxidizing current. The resulting static ORP (sORP) measurement displayed reflects the average of the final ten (10) seconds (or twenty [20] readings) of the run. The sample analysis is completed in approximately 3 min. Displayed sORP values above the normal range imply an imbalance between oxidants and antioxidants (elevated oxidants) and signal the presence of oxidative stress in the specimen.



Figure 12. MiOXSYS® System showing the analyzer, calibration card and sensors (Reprinted with permission from Aytu Bioscience, Englewood, CO, USA).

3.4.16.2. Procedure

A sensor strip was inserted into the machine and 20 μL of spent medium were added to the opening on the sensor strip. Once the sample filled the reference electrode, thereby completing the electrochemical circuit, the test was started. After about 4 min, the ORP was reported as static ORP (sORP) measure in millivolts (mV), which is the integrated measure of the existing balance between total oxidants and reductants in the sample. This was done for each spent medium sample using a new sensor strip and pipet tip each time. Measurements were taken for spent culture media from wells that contained embryos and also blank samples from wells that did not have embryos, which served as controls.

3.5. STATISTICAL ANALYSIS

All statistical testing was performed using MedCalc Statistical Software version 19.1 (MedCalc Software bv, Ostend, Belgium; <https://www.medcalc.org>; 2019). The data was analyzed for normal distribution using the D'Agostino- Pearson test, and was found not be normally distributed. Outliers were detected using a Tukey test and excluded from the analysis.

Since the data was not normally distributed, comparison testing of various parameters measured was done using a Mann-Whitney test. Comparison of percentages were done using a Chi-squared test. A p-value of <0.05 was considered to be statistically significant.

4. RESULTS

4.1. Comparison of human blastocyst development using sequential media versus One-Step™ media in EmbryoScope™ and Planer incubators

A total of 140 patients with a total of 1,749 fertilized eggs and their subsequent blastocyst development were retrospectively analyzed (Table 1a). There were no significant differences between patient ages in any of the groups compared. Irrespective of the incubation system used, there were no significant differences in fertilization rate after ICSI in eggs cultured in sequential media or One-Step™ medium (81.8% vs 78.9%, $p>0.1271$). Furthermore, no significant differences were noted in fertilization rate in sequential media compared to One-Step™ medium in either of the EmbryoScope™ (81.3% vs 78.7%, $p>0.2907$) or the Planer incubators (82.3% vs 80.8%, $p>0.7547$).

The total blastocyst development rate (Day 5 and Day 6) was significantly higher in embryos cultured in One-Step™ medium when compared to sequential media (62.2 % vs 54.5%; $p=0.0031$). One-Step™ medium also showed higher blastocyst rates on Day 5 (34.3% vs 29.6%, $p>0.0591$) and on Day 6 (27.8% vs 24.8%, $p>0.2021$), but these did not significantly differ.

When comparing the efficiency of the incubation systems (Table 1b), the EmbryoScope™ incubator yielded significantly higher total blastocyst rates (Day 5 + Day 6) than embryos cultured in the Planer incubator (60.6% vs 52.8%, $p=0.005$). With regards to the culture medium, the EmbryoScope™ still yielded higher blastocyst rates than the Planers when

using either sequential media (56.9% vs 52.4%; $p=0.2167$) or One-Step™ medium (62.8%vs 55.9%, $p=0.2977$), but these were not statistically significant. Furthermore, the Day 5 blastocyst rates were also significantly higher in the EmbryoScope™ culture (34.0% vs 27.5%, $p=0.0132$). However, no significant differences in blastocyst rates were noted in the EmbryoScope™ on Day 6 compared to Planer incubation (26.7% vs 25.4%, $p=0.6000$).



Table 1a. Comparison of human blastocyst development using sequential media (SM) versus One-Step™ medium (OSM) in EmbryoScope™ (ES) and Planer (PL) incubators

	Sequential Media (SM)			One-Step™ Media (OSM)			p-value		
	ES	Planer	Total	ES	Planer	Total	ES (SM) v ES (OSM)	PL (SM) v PL (OSM)	Total (SM) v Total (OSM)
Patients (n)	29	44	73	62	5	67	N/A	N/A	N/A
Mean patient age (yrs)	32.4	32.1	32.2	31.0	36.3	31.7	0.7108	0.1192	0.3202
Eggs retrieved (n)	512	640	1152	948	87	1035	N/A	N/A	N/A
Mature eggs injected (n)	417	508	925	751	73	824	N/A	N/A	N/A
Egg maturation rate (%)	81.4	79.3	80.3	79.2	83.9	79.6	0.3164	0.3159	0.6831
Fertilized eggs (2PN) (n)	339	418	757	591	59	650	N/A	N/A	N/A
Fertilization rate (%)	81.3	82.3	81.8	78.7	80.8	78.9	0.2907	0.7547	0.1271
Blastocysts – Day 5 (n)	108	116	224	208	15	223	N/A	N/A	N/A
Blastocysts – Day 6 (n)	85	103	188	163	18	181	N/A	N/A	N/A
Total Blastocysts – Day 5+6 (n)	193	219	412	371	33	404	N/A	N/A	N/A
Blastocyst rate (BR) – Day 5 (%)	31.9	27.8	29.6	35.2	25.4	34.3	0.3110	0.6994	0.0591
Blastocyst rate (BR)– Day 6 (%)	25.1	24.6	24.8	27.6	30.5	27.8	0.4112	0.3298	0.2021
Total BR – Day 5+6 (%)	56.9	52.4	54.4	62.8	55.9	62.2	0.0790	0.6145	0.0031

Patient age was compared with Mann-Whitney test. Egg maturation rate, fertilization rate, and blastocyst rates was compared with Chi-squared test.

Table 1b. Comparison of human blastocyst development using EmbryoScope™ (ES) versus Planer (PL) incubators in both sequential media (SM) and One-Step™ medium (OSM).

	EmbryoScope™ (ES)			Planer (PL)			p-value		
	SM	OSM	Total	SM	OSM	Total	SM (ES) v SM (PL)	OSM(ES) v OSM (PL)	Total (ES) v Total (PL)
Fertilized eggs (2PN) (n)	339	591	930	418	59	477	N/A	N/A	N/A
Blastocyst rate – Day 5 (%)	31.9	35.2	34.0	27.8	25.4	27.5	0.2196	0.1309	0.0132
Blastocyst rate – Day 6 (%)	25.1	27.6	26.7	24.6	30.5	25.4	0.8743	0.6359	0.6000
Total Blastocyst – Day 5+6 (%)	56.9	62.8	60.6	52.4	55.9	52.8	0.2167	0.2977	0.0050

Chi-squared test.

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4.2. Comparison of blastocyst development and time-lapse morphokinetics of mouse embryos cultured in One-Step™ medium supplemented with IGF-1 of insulin.

Using the hybrid mouse strain (B6D2F1 & B6C3F1), no significant differences were noted for any of the time points analyzed (Table 2a) between embryos cultured in IGF-1 and controls. However, insulin cultured embryos showed slower development as evidenced by the significantly later start of blastulation ($57.72 \pm 3.74\text{hr}$ vs $56.72 \pm 3.84\text{hr}$, $p=0.0348$) compared to controls (Table 2a).

Furthermore, insulin cultured embryos showed lower blastocyst rates (94.3% vs 99.1%, $p=0.0512$) and expanded blastocyst rates (91.4% vs 99.1%, $p=0.0086$) compared to controls (Table 2b). There were no significant differences in blastocyst development rates (96.2% vs 99.1%, $p=0.1648$) and expanded blastocyst rates (96.2% vs 99.1%, $p=0.1648$) between embryos cultured in IGF-1 and controls.

When using C57BL-6N mouse strain, time-lapse morphokinetics of insulin cultured embryos initially showed a significantly slower time to morula than controls ($49.03 \pm 4.46\text{hr}$ vs 48.06 ± 3.95 ; $p=0.0445$) but faster time to blastocyst ($70.59 \pm 8.3\text{hr}$ vs 72.60 ± 8.67 ; $p=0.0383$) (Table 2c). In addition, blastocyst development was significantly lower than controls (70.8% vs 81.4%, $p=0.0313$) (Table 2d). Again, no significant differences between time-lapse morphokinetics (Table 2c) as well as blastocyst and expanded blastocyst development rates (Table 2d) were noted between embryos cultured in IGF-1 and controls.

Table 2a. Comparison of time-lapse morphokinetics between mouse embryos (hybrid) cultured in One-Step™ medium, IGF-1 and insulin.

Time points	B6D2F1 & B6C3F1 Hybrid mouse strain			p-value		
	Control	IGF-1	Insulin	Control vs IGF-1	Control vs Insulin	IGF-1 vs Insulin
t3	19.69 ± 2.37 (n=105)	19.97 ± 1.44 (n=101)	19.77 ± 2.52 (n=99)	0.9636	0.1706	0.3007
t4	20.98 ± 2.73 (n=105)	20.91 ± 2.10 (n=101)	21.01 ± 1.86 (n=99)	0.9171	0.1788	0.2636
t5	29.48 ± 1.88 (n=104)	29.66 ± 1.96 (n=101)	29.61 ± 1.96 (n=99)	0.4654	0.4137	0.9815
t6	29.91 ± 2.01 (n=103)	30.04 ± 2.14 (n=100)	30.12 ± 1.89 (n=99)	0.6344	0.1551	0.4513
t7	30.92 ± 2.51 (n=102)	30.90 ± 2.30 (n=98)	30.82 ± 1.78 (n=97)	0.6760	0.3558	0.8114
t8	30.86 ± 2.05 (n=96)	30.97 ± 2.25 (n=97)	30.96 ± 1.80 (n=95)	0.6744	0.2367	0.6207
tSC	35.23 ± 2.88 (n=105)	35.18 ± 2.74 (n=101)	35.12 ± 2.10 (n=99)	0.9357	0.6393	0.7778
tM	41.54 ± 3.67 (n=105)	41.93 ± 3.09 (n=101)	41.96 ± 2.99 (n=99)	0.2336	0.1003	0.5766
tSB	56.72 ± 3.84 ^a (n=105)	56.66 ± 3.69 (n=101)	57.72 ± 3.74 ^a (n=99)	0.8636	0.0348	0.0273
tB	63.41 ± 5.94 (n=105)	62.29 ± 4.61 (n=101)	63.14 ± 4.79 (n=99)	0.5030	0.5133	0.0973
tEB	69.71 ± 7.60 (n=105)	68.83 ± 6.62 (n=101)	69.29 ± 6.37 (n=96)	0.6988	0.9092	0.6206
tHB	79.67 ± 8.55 (n=98)	79.63 ± 7.48 (n=94)	80.42 ± 8.67 (n=96)	0.9069	0.4771	0.4713

Mann-Whitney test

Table 2b. Blastocyst and expanded blastocyst development rate of hybrid mouse embryos cultured in One-Step™ medium, IGF-1 and insulin.

	B6D2F1 & B6C3F1 Hybrid mouse strain			p-value		
	Control	IGF-1	Insulin	Control vs IGF-1	Control vs Insulin	IGF-1 vs Insulin
Embryos (n)	106	105	105	N/A	N/A	N/A
Blastocysts (n)	105	101	99	N/A	N/A	N/A
Expanded blastocysts (n)	105	101	96	N/A	N/A	N/A
Blastocyst rate (%)	99.1	96.2	94.3	0.1648	0.0512	0.5185
Expanded blastocyst rate (%)	99.1	96.2	91.4	0.1648	0.0086	0.1502

Chi-squared test. Blastocysts are defined as having 50% blastulation. Expanded blastocysts are defined as having 100% blastulation and zona pellucida thinning

Table 2c. Comparison of time-lapse morphokinetics between mouse embryos (C57BL) cultured in One-Step™ medium, IGF-1 and insulin.

Time points	C57BL-6N mouse strain			p-value		
	Control	IGF-1	Insulin	Control vs IGF-1	Control vs Insulin	IGF-1 vs Insulin
t3	20.83 ± 4.69 (n=127)	20.60 ± 5.43 (n=110)	20.40 ± 5.43 (n=102)	0.3317	0.8237	0.4583
t4	24.17 ± 5.23 (n=127)	24.31 ± 6.17 (n=110)	24.70 ± 6.12 (n=102)	0.9349	0.2164	0.2811
t5	32.94 ± 2.43 (n=126)	33.23 ± 2.57 (n=106)	33.19 ± 2.49 (n=100)	0.4821	0.3762	0.8571
t6	33.65 ± 2.67 (n=122)	34.18 ± 3.58 (n=104)	33.97 ± 2.85 (n=94)	0.4501	0.3954	0.9446
t7	35.06 ± 3.29 (n=116)	35.19 ± 3.30 (n=88)	35.15 ± 2.96 (n=85)	0.4965	0.4336	0.8937
t8	35.56 ± 3.67 (n=107)	35.38 ± 3.01 (n=79)	35.67 ± 3.22 (n=81)	0.7988	0.3511	0.5200
tSC	42.08 ± 4.64 (n=127)	41.67 ± 4.43 (n=110)	40.75 ± 3.48 (n=102)	0.5534	0.0584	0.2306
tM	48.06 ± 3.95 (n=125)	49.24 ± 4.79 (n=110)	49.03 ± 4.46 (n=102)	0.0530	0.0445	0.9126
tSB	64.24 ± 6.48 (n=127)	65.07 ± 7.19 (n=110)	63.24 ± 5.78 (n=102)	0.4928	0.2628	0.1052
tB	72.60 ± 8.67 (n=127)	71.79 ± 8.88 (n=110)	70.59 ± 8.30 (n=102)	0.3618	0.0383	0.3737
tEB	81.49 ± 9.24 (n=115)	81.60 ± 10.84 (n=107)	79.24 ± 10.20 (n=95)	0.9076	0.0720	0.1213
tHB	96.79 ± 8.10 (n=89)	95.08 ± 9.74 (n=68)	95.74 ± 9.15 (n=68)	0.4699	0.6656	0.7342

Mann-Whitney test.

Table 2d. Blastocyst and expanded blastocyst development rate of C57BL mouse embryos cultured in One-Step™ medium, IGF-1 and insulin.

	C57BL-6N mouse strain			p-value		
	Control	IGF-1	Insulin	Control vs IGF-1	Control vs Insulin	IGF-1 vs Insulin
Embryos (n)	156	144	144	N/A	N/A	N/A
Blastocysts (n)	127	110	102	N/A	N/A	N/A
Expanded blastocysts (n)	115	107	95	N/A	N/A	N/A
Blastocyst rate (%)	81.4	76.4	70.8	0.2889	0.0313	0.2819
Expanded blastocyst rate (%)	73.7	74.3	66.0	0.9059	0.1466	0.1244

Chi-squared test. Blastocysts are defined as having 50% blastulation. Expanded blastocysts are defined as having 100% blastulation and zona pellucida thinning.

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4.3. Comparison of blastocyst development and time-lapse morphokinetics of mouse embryos cultured in a low oxygen (2%) environment.

Embryo morphokinetics in the hybrid mouse model showed significantly slower embryo development in 2% oxygen environment in all time points after the 8-cell stage (from start of compaction up to hatching blastocyst) when compared to 6% oxygen (Table 3a).

Table 3a. Comparison of time-lapse morphokinetics between mouse embryos (hybrid) cultured in 6% oxygen versus 2% oxygen.

Time points	B6D2F1 & B6C3F1 Hybrid mouse strain		
	6% Oxygen	2% Oxygen	p-value
t3	19.69 ± 2.37 (n=105)	19.95 ± 1.76 (n=105)	0.2353
t4	20.98 ± 2.73 (n=105)	20.95 ± 1.68 (n=105)	0.0650
t5	29.48 ± 1.88 (n=104)	29.34 ± 1.62 (n=105)	0.5643
t6	29.91 ± 2.01 (n=103)	29.78 ± 1.95 (n=104)	0.5602
t7	30.92 ± 2.51 (n=102)	30.90 ± 2.42 (n=104)	0.9832
t8	30.86 ± 2.05 (n=96)	31.56 ± 2.89 (n=102)	0.0645
tSC	35.23 ± 2.88 (n=105)	36.05 ± 3.09 (n=105)	0.0112
tM	41.54 ± 3.67 (n=105)	42.81 ± 3.31 (n=104)	<0.0001
tSB	56.72 ± 3.84 (n=105)	59.14 ± 5.62 (n=105)	0.0015
tB	63.41 ± 5.94 (n=105)	68.63 ± 7.69 (n=105)	<0.0001
tEB	69.71 ± 7.60 (n=105)	77.62 ± 9.82 (n=103)	<0.0001
tHB	79.67 ± 8.55 (n=98)	85.99 ± 8.60 (n=91)	<0.0001

Mann-Whitney test

Fluorescence microscopy with DAPI was able to stain the cells well enough for analysis and was comparable to images seen in the EmbryoScope™ (Figure 13). Blastocysts cultured at 2% oxygen showed a significantly lower cell counts than those cultured at 6% oxygen (Table 3b; Figure 14).

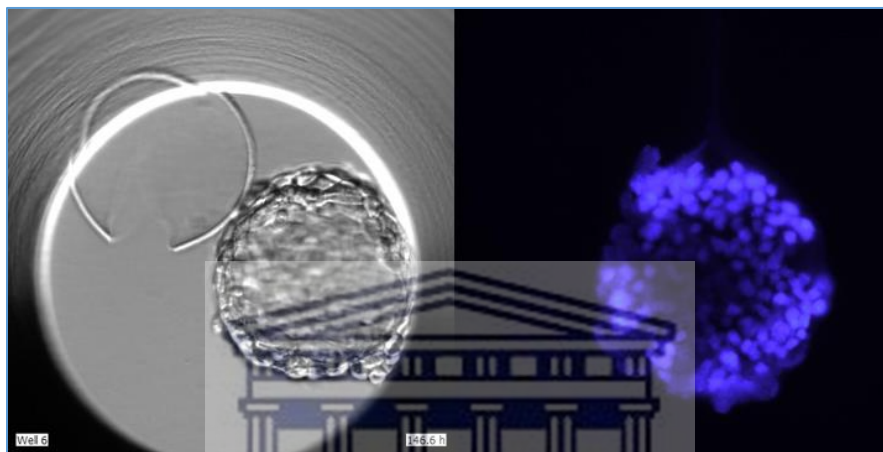


Figure 13. Blastocyst imaging from EmbryoScope™ and fluorescence microscopy.

Table 3b. Cell counts of blastocysts cultured in 6% oxygen versus 2% oxygen

	6% Oxygen (n=20)	2% Oxygen (n=18)	p-value
Cell counts	83.9 ± 26.1	65.0 ± 10.3	0.0062

Mann-Whitney test.

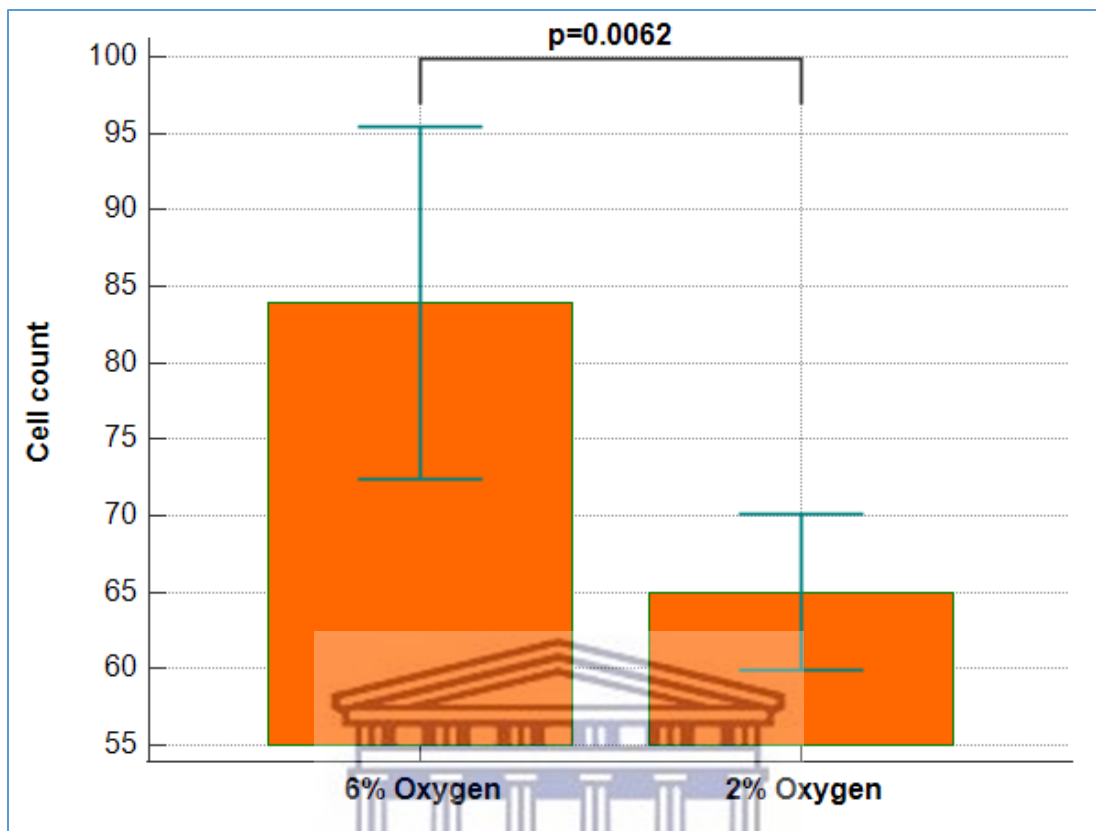


Figure 14. Cell counts of blastocysts cultured at 6% oxygen versus 2% oxygen showing 2% O₂ resulting in significantly lower number of cells ($p=0.0062$; Mann-Whitney test)

Similar trends were noted in the C57BL-6N mouse strain as well, but the slower embryo development in 2% O₂ culture only reached significant differences in the time to reach morula (tM) and expanded blastocyst (tEB) stages (Table 3c).

Table 3c. Comparison of time-lapse morphokinetics between mouse embryos (C57BL) cultured in 6% oxygen versus 2% oxygen.

Time points	C57BL-6N mouse strain		
	6% Oxygen	2% Oxygen	p-value
t3	20.83 ± 4.69 (n=127)	19.60 ± 5.76 (n=90)	0.1421
t4	24.17 ± 5.23 (n=127)	24.19 ± 6.12 (n=90)	0.6339
t5	32.94 ± 2.43 (n=126)	32.53 ± 3.97 (n=88)	0.1429
t6	33.65 ± 2.67 (n=122)	33.61 ± 4.56 (n=86)	0.3351
t7	35.06 ± 3.29 (n=116)	34.78 ± 4.54 (n=78)	0.3465
t8	35.56 ± 3.67 (n=107)	35.41 ± 4.93 (n=74)	0.5541
tSC	42.08 ± 4.64 (n=127)	42.44 ± 5.33 (n=90)	0.3096
tM	48.06 ± 3.95 (n=125)	49.31 ± 5.30 (n=90)	0.0396
tSB	64.24 ± 6.48 (n=127)	65.61 ± 7.13 (n=90)	0.1506
tB	72.60 ± 8.67 (n=127)	74.99 ± 10.34 (n=90)	0.1131
tEB	81.49 ± 9.24 (n=115)	85.44 ± 11.77 (n=82)	0.0193
tHB	96.79 ± 8.10 (n=89)	97.66 ± 9.47 (n=34)	0.4351

Mann-Whitney test.

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Even though the 2% O₂ culture resulted in slower embryo development, there was no significant differences noted in the blastocyst (97.2% vs 99.1%, p=0.3047) and expanded blastocyst (95.4% vs 99.1%, p=0.0998) development rates for the hybrid model in 2% O₂ when compared to 6% O₂ culture (Table 3d). However, using the C57BL-6N model, the blastocyst development rates were significantly decreased in the 2% O₂ group compared to 6% O₂ group (62.5% vs 81.4%; p=0.0003) as well as the expanded blastocyst development rate (56.0% vs 73.7%; p=0.0022).

Table 3d. Blastocyst and expanded blastocyst development rate of two different strains of mouse embryos cultured in 6% oxygen versus 2% oxygen.

	B6D2F1 & B6C3F1 Hybrid			C57BL-6N		
	6% O ₂	2% O ₂	p-value	6% O ₂	2% O ₂	p-value
Embryos (n)	106	108	N/A	156	144	N/A
Blastocysts (n)	105	105	N/A	127	90	N/A
Expanded blastocysts (n)	105	103	N/A	115	82	N/A
Blastocyst rate (%)	99.1	97.2	0.3047	81.4	62.5	0.0003
Expanded blastocyst rate (%)	99.1	95.4	0.0998	73.7	56.0	0.0022

Chi-squared test. Blastocysts are defined as having 50% blastulation. Expanded blastocysts are defined as having 100% blastulation and zona pellucida thinning.

4.4. Evaluation of a different strain of mouse embryos as a better model for research and quality control.

Comparing the time-lapse morphokinetics of hybrid mouse embryos to C57BL-6N embryos in various culture conditions, the C57BL-6N strain of mouse embryos showed significantly slower embryo development ($p < 0.0001$) in all time points measured when cultured in One-Step™ medium at 6% O₂ (Table 4a), One-Step™ medium with IGF-1 (Table 4b), One-Step™ medium with insulin (Table 4c), and One-Step™ medium at 2% O₂ (Table 4d).

Table 4a. Comparison of time-lapse morphokinetics of two different strains of mouse embryos (B6D2F1 & B6C3F1 Hybrid versus C57BL-6N) cultured in One-Step™ medium.

Time points	One-Step™ (6% O ₂)		
	Hybrid	C57BL	p-value
t3 (hr)	19.69 ± 2.37 (n=105)	20.83 ± 4.69 (n=127)	<i>p</i> <0.0001
t4 (hr)	20.98 ± 2.73 (n=105)	24.17 ± 5.23 (n=127)	<i>p</i> <0.0001
t5 (hr)	29.48 ± 1.88 (n=104)	32.94 ± 2.43 (n=126)	<i>p</i> <0.0001
t6 (hr)	29.91 ± 2.01 (n=103)	33.65 ± 2.67 (n=122)	<i>p</i> <0.0001
t7 (hr)	30.92 ± 2.51 (n=102)	35.06 ± 3.29 (n=116)	<i>p</i> <0.0001
t8 (hr)	30.86 ± 2.05 (n=96)	35.56 ± 3.67 (n=107)	<i>p</i> <0.0001
tSC (hr)	35.23 ± 2.88 (n=105)	42.08 ± 4.64 (n=127)	<i>p</i> <0.0001
tM (hr)	41.54 ± 3.67 (n=105)	48.06 ± 3.95 (n=125)	<i>p</i> <0.0001
tSB (hr)	56.72 ± 3.84 (n=105)	64.24 ± 6.48 (n=127)	<i>p</i> <0.0001
tB (hr)	63.41 ± 5.94 (n=105)	72.60 ± 8.67 (n=127)	<i>p</i> <0.0001
tEB (hr)	69.71 ± 7.60 (n=105)	81.49 ± 9.24 (n=115)	<i>p</i> <0.0001
tHB (hr)	79.67 ± 8.55 (n=98)	96.79 ± 8.10 (n=89)	<i>p</i> <0.0001

Mann-Whitney test.

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Table 4b. Comparison of time-lapse morphokinetics of two different strains of mouse embryos (B6D2F1 & B6C3F1 Hybrid versus C57BL-6N) cultured in One-Step™ medium with IGF-1

Time points	IGF-1 (6% O ₂)		
	Hybrid	C57BL	p-value
t3 (hr)	19.97 ± 1.44 (n=101)	20.60 ± 5.43 (n=110)	<i>p</i> <0.0001
t4 (hr)	20.91 ± 2.10 (n=101)	24.31 ± 6.17 (n=110)	<i>p</i> <0.0001
t5 (hr)	29.66 ± 1.96 (n=101)	33.23 ± 2.57 (n=106)	<i>p</i> <0.0001
t6 (hr)	30.04 ± 2.14 (n=100)	34.18 ± 3.58 (n=104)	<i>p</i> <0.0001
t7 (hr)	30.90 ± 2.30 (n=98)	35.19 ± 3.30 (n=88)	<i>p</i> <0.0001
t8 (hr)	30.97 ± 2.25 (n=97)	35.38 ± 3.01 (n=79)	<i>p</i> <0.0001
tSC (hr)	35.18 ± 2.74 (n=101)	41.67 ± 4.43 (n=110)	<i>p</i> <0.0001
tM (hr)	41.93 ± 3.09 (n=101)	49.24 ± 4.79 (n=110)	<i>p</i> <0.0001
tSB (hr)	56.66 ± 3.69 (n=101)	65.07 ± 7.19 (n=110)	<i>p</i> <0.0001
tB (hr)	62.29 ± 4.61 (n=101)	71.79 ± 8.88 (n=110)	<i>p</i> <0.0001
tEB (hr)	68.83 ± 6.62 (n=101)	81.60 ± 10.84 (n=107)	<i>p</i> <0.0001
tHB (hr)	79.63 ± 7.48 (n=94)	95.08 ± 9.74 (n=68)	<i>p</i> <0.0001

Mann-Whitney test.

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Table 4c. Comparison of time-lapse morphokinetics of two different strains of mouse embryos (B6D2F1 & B6C3F1 Hybrid versus C57BL-6N) cultured in One-Step™ medium with insulin.

Time points	Insulin (6% O ₂)		
	Hybrid	C57BL	p-value
t3 (hr)	19.77 ± 2.52 (n=99)	20.40 ± 5.43 (n=102)	<i>p</i> <0.0001
t4 (hr)	21.01 ± 1.86 (n=99)	24.70 ± 6.12 (n=102)	<i>p</i> <0.0001
t5 (hr)	29.61 ± 1.96 (n=99)	33.19 ± 2.49 (n=100)	<i>p</i> <0.0001
t6 (hr)	30.12 ± 1.89 (n=99)	33.97 ± 2.85 (n=94)	<i>p</i> <0.0001
t7 (hr)	30.82 ± 1.78 (n=97)	35.15 ± 2.96 (n=85)	<i>p</i> <0.0001
t8 (hr)	30.96 ± 1.80 (n=95)	35.67 ± 3.22 (n=81)	<i>p</i> <0.0001
tSC (hr)	35.12 ± 2.10 (n=99)	40.75 ± 3.48 (n=102)	<i>p</i> <0.0001
tM (hr)	41.96 ± 2.99 (n=99)	49.03 ± 4.46 (n=102)	<i>p</i> <0.0001
tSB (hr)	57.72 ± 3.74 (n=99)	63.24 ± 5.78 (n=102)	<i>p</i> <0.0001
tB (hr)	63.14 ± 4.79 (n=99)	70.59 ± 8.30 (n=102)	<i>p</i> <0.0001
tEB (hr)	69.29 ± 6.37 (n=96)	79.24 ± 10.20 (n=95)	<i>p</i> <0.0001
tHB (hr)	80.42 ± 8.67 (n=96)	95.74 ± 9.15 (n=68)	<i>p</i> <0.0001

Mann-Whitney test.

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Table 4d. Comparison of time-lapse morphokinetics of two different strains of mouse embryos (B6D2F1 & B6C3F1 Hybrid versus C57BL-6N) cultured in One-Step™ medium at 2% oxygen.

Time points	One-Step™ (2% O ₂)		
	Hybrid	C57BL	p-value
t3 (hr)	19.95 ± 1.76 (n=105)	19.60 ± 5.76 (n=90)	<i>p</i> <0.0001
t4 (hr)	20.95 ± 1.68 (n=105)	24.19 ± 6.12 (n=90)	<i>p</i> <0.0001
t5 (hr)	29.34 ± 1.62 (n=105)	32.53 ± 3.97 (n=88)	<i>p</i> <0.0001
t6 (hr)	29.78 ± 1.95 (n=104)	33.61 ± 4.56 (n=86)	<i>p</i> <0.0001
t7 (hr)	30.90 ± 2.42 (n=104)	34.78 ± 4.54 (n=78)	<i>p</i> <0.0001
t8 (hr)	31.56 ± 2.89 (n=102)	35.41 ± 4.93 (n=74)	<i>p</i> <0.0001
tSC (hr)	36.05 ± 3.09 (n=105)	42.44 ± 5.33 (n=90)	<i>p</i> <0.0001
tM (hr)	42.81 ± 3.31 (n=104)	49.31 ± 5.30 (n=90)	<i>p</i> <0.0001
tSB (hr)	59.14 ± 5.62 (n=105)	65.61 ± 7.13 (n=90)	<i>p</i> <0.0001
tB (hr)	68.63 ± 7.69 (n=105)	74.99 ± 10.34 (n=90)	<i>p</i> <0.0001
tEB (hr)	77.62 ± 9.82 (n=103)	85.44 ± 11.77 (n=82)	<i>p</i> <0.0001
tHB (hr)	85.99 ± 8.60 (n=91)	97.66 ± 9.47 (n=34)	<i>p</i> <0.0001

Mann-Whitney test.

Furthermore, the C57BL-6N strain of mouse embryos showed significantly lower blastocyst rates and expanded blastocyst rates in each of the four culture conditions (Table 4e). When combining all four culture systems, the total blastocyst rate was significantly lower in the C57BL-6N group (73.0% vs 96.7%; *p*<0.0001) as well as the expanded blastocyst rate (67.9% vs 95.5%; *p*<0.0001) compared to the hybrid strain.

Table 4e. Comparison of blastocyst and expanded blastocyst development rates of two different strains of mouse embryos (B6D2F1 & B6C3F1 Hybrid versus C57BL-6N) in four different culture conditions (One-Step medium, IGF-1, insulin and 2% oxygen).


Culture	Mouse strain	Embryos (n)	Blastocysts (n)	Expanded Blastocysts (n)	Blastocyst Rate (%)	p-value (Blastocyst Rate)	Expanded Blastocyst Rate (%)	p-value (Expanded Blastocyst Rate)
One-Step™	Hybrid	106	105	105	99.1	<0.0001	99.1	<0.0001
	C57BL	156	127	115	81.4		73.7	
IGF-1	Hybrid	105	101	101	96.2	<0.0001	96.2	<0.0001
	C57BL	144	110	107	76.4		74.3	
Insulin	Hybrid	105	99	96	94.3	<0.0001	91.4	<0.0001
	C57BL	144	102	95	70.8		66.0	
2% O₂	Hybrid	108	105	103	97.2	<0.0001	95.4	<0.0001
	C57BL	144	90	82	62.5		56.9	
TOTAL	Hybrid	424	410	405	96.7	<0.0001	95.5	<0.0001
	C57BL	588	429	399	73.0		67.9	

Chi-squared test. Blastocysts are defined as having 50% blastulation. Expanded blastocysts are defined as having 100% blastulation and zona pellucida thinning.

4.5. GDF-9 levels in spent media and pregnancy outcome.

In this study, the GDF-9 levels in spent media of embryos on Day 5 and Day 6 that had subsequent frozen embryo transfers and pregnancy rates were compared. There was a significant difference in biochemical (80.7% vs 62.5%; $p=0.0326$) and clinical pregnancy rates (68.4% vs 48.2%; $p=0.0302$) between Day 5 and Day 6 embryos. This was accompanied with significantly higher GDF-9 levels ($6,456.6 \pm 3,851.6$ pg/mL vs $3,933.6 \pm 2,705.9$ pg/mL; $p=0.0002$) in Day 5 spent medium compared to Day 6 (Table 5a). This significant difference in GDF-9 is further illustrated in Figure 15.

Table 5a. Pregnancy outcome and GDF-9 levels in spent culture medium of embryos that were frozen on Day 5 and Day 6.



	Day 5	Day 6	p-value
FET's (n)	57	56	N/A
Biochemical pregnancy (n)	46	35	N/A
Biochemical pregnancy (%)	80.7	62.50	0.0326
Clinical pregnancy (n)	39	27	N/A
Clinical pregnancy (%)	68.4	48.21	0.0302
GDF-9 (pg/mL)	$6,456.6 \pm 3,851.6$	$3,933.6 \pm 2,705.9$	0.0002

Pregnancy rates compared with chi-squared test. GDF-9 values compared with Mann-Whitney test. Values expressed as mean \pm SD

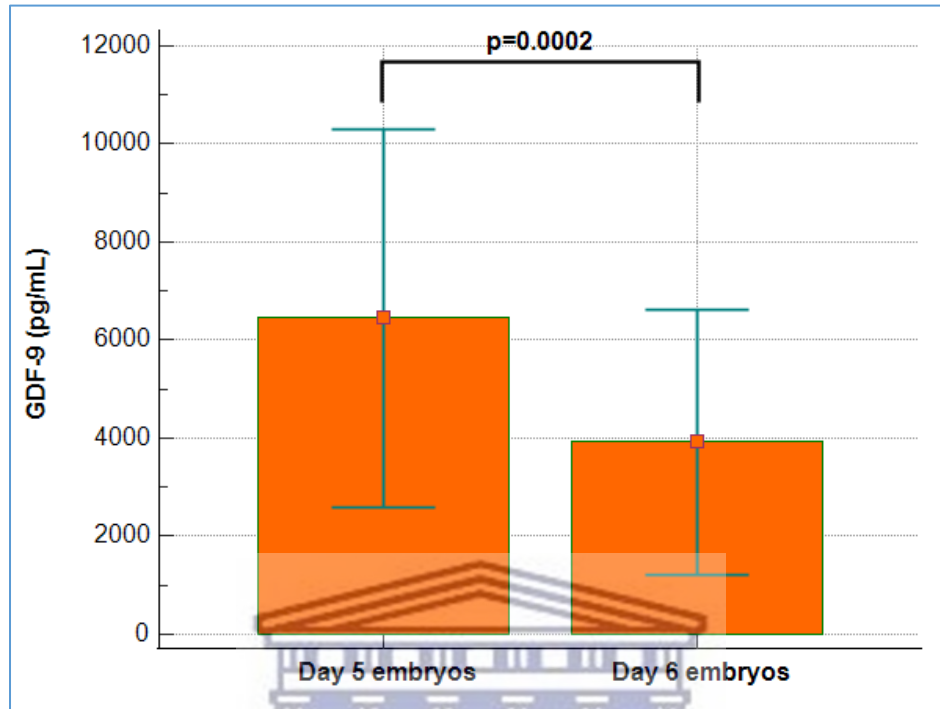


Figure 15. GDF-9 values in spent medium of Day 5 versus Day 6 embryos showing D6 spent medium having significantly lower GDF-9 ($p=0.0002$, Mann-Whitney test)

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When further categorizing the pregnancy group into those that showed no pregnancy (negative β -hCG 2 weeks post transfer), biochemical pregnancy (positive β -hCG after 2 weeks post transfer), clinical pregnancy (biochemical pregnancy with heartbeat 6 weeks post transfer), and miscarriage (biochemical pregnancy with no heartbeat 6 weeks post transfer), there were no significant differences in GDF-9 values between these groups on either Day 5 or Day 6 (Table 5b). However, the GDF-9 values on Day 5 continued to be significantly higher than D6 in the biochemical ($6,540.9 \pm 3,826.9$ pg/mL vs $3,827.6 \pm 2,777.9$ pg/mL; $p=0.0007$) and clinical pregnancy groups ($6,358.4 \pm 3,631$ pg/mL vs $3,319.8 \pm 2,333.7$ pg/mL; $p=0.0002$).

Table 5b. GDF-9 levels in spent culture medium between embryos from pregnant versus non-pregnant cycles grouped by day of embryo cryopreservation.

	Day 5 embryo	Day 6 embryo	Total	D5 vs D6 p-value
No pregnancy (negative β -hCG)	6,103.9 \pm 4,123.3 (n=11)	4,110.4 \pm 2638.9 (n=21)	4,795.7 \pm 3,301.9 (n=32)	0.2418
Biochemical pregnancy (positive β -hCG)	6,540.9 \pm 3,826.9 (n=46)	3,827.6 \pm 2,777.9 (n=35)	5,368.5 \pm 3,653.3 (n=81)	0.0007
Clinical pregnancy (heartbeat)	6,358.4 \pm 3,631 (n=39)	3,319.8 \pm 2,333.7 (n=27)	5,115.4 \pm 3,486.3 (n=66)	0.0002
Miscarriage (no heartbeat)	7,557.7 \pm 4,989.8 (n=7)	5,541.3 \pm 3,591.8 (n=8)	6,482.3 \pm 4,266.8 (n=15)	0.4875
Total	6,456.6 \pm 3,851.6 (n=57)	3,933.6 \pm 2,705.9 (n=56)	5,206.3 \pm 3,552.2 (n=113)	<0.0002

Mann-Whitney test. Values expressed as mean \pm SD. No pregnancy: negative β -HCG, 2 weeks post transfer.

Biochemical pregnancy: positive β -HCG, 2 weeks post transfer. Clinical pregnancy: Biochemical pregnancy with

heartbeat 6 weeks post transfer. Miscarriage: biochemical pregnancy with no heartbeat 6 weeks post transfer.

Time-lapse morphokinetics analysis showed no significant difference in any of the time points evaluated for Day 5 embryos between the various pregnancy groups (Table 5c) with the exception of time to blastocyst (tB) between the non-pregnant and clinical pregnancy groups (102.37 ± 6.95 vs 97.95 ± 5.69 , $p=0.0494$), even though this difference was very slightly significant, indicating that TLM could not differentiate between embryos that were more likely to implant than those that were not.

For Day 6 embryos, there were significant differences in TLM in the non-pregnant group compared to the biochemical and clinical pregnancy groups, starting as early as the time point for the 2 pronuclei to fade (syngamy) up until the 8-cell stage and then again at the start of blastulation (Table 5d). Interestingly, the biochemical and clinical pregnancy groups showed slower embryo development for these time points, suggesting that faster embryo development might not be the best predictor of implantation potential.

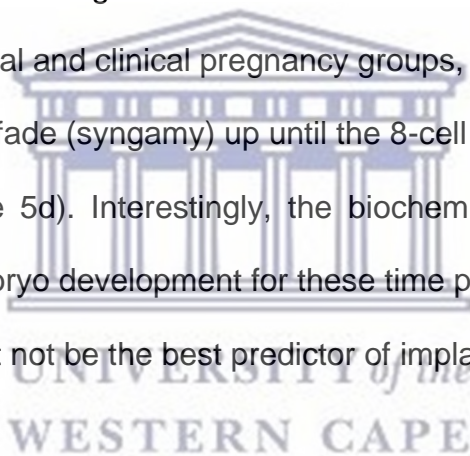


Table 5c. Time-lapse morphokinetics and GDF-9 measurements in spent medium of Day 5 embryos according to pregnancy outcome.

DAY 5								
	No pregnancy (NP) (N=11)	Biochemical Pregnancies (BP)			p-values			
		Miscarriage (MC) (N=7)	Clinical (CP) (n=39)	Total BP (n=46)	NP vs MC	NP vs CP	NP vs BP	MC vs CP
tPNa (hr)	8.97 ± 2.59	7.34 ± 1.55	7.81 ± 2.19	7.74 ± 2.10	0.1791	0.0935	0.0779	0.9501
tPNf (hr)	23.24 ± 2.35	23.32 ± 2.82	22.65 ± 2.30	22.75 ± 2.36	0.9298	0.5177	0.5567	0.5945
t2 (hr)	25.63 ± 2.40	25.97 ± 3.09	25.03 ± 2.44	25.17 ± 2.54	1.0000	0.5196	0.5851	0.4177
t3 (hr)	36.52 ± 3.25	37.04 ± 4.28	35.27 ± 3.92	35.52 ± 3.97	0.9546	0.4801	0.5534	0.5054
t4 (hr)	36.58 ± 4.97	37.63 ± 4.38	36.56 ± 3.24	36.73 ± 3.40	0.9298	0.5821	0.6564	0.5925
t5 (hr)	48.27 ± 4.11	47.95 ± 7.33	48.18 ± 4.57	48.15 ± 4.99	1.0000	0.9569	0.9537	0.7781
t6 (hr)	47.82 ± 6.61	50.36 ± 5.24	49.48 ± 4.87	49.62 ± 4.88	0.4789	0.6151	0.5417	0.6189
t7 (hr)	51.53 ± 4.27	51.72 ± 6.29	51.19 ± 5.63	51.27 ± 5.65	1.0000	0.6312	0.6769	0.8537
t8 (hr)	52.37 ± 7.75	52.23 ± 6.01	53.28 ± 6.97	53.11 ± 6.77	0.9298	0.6662	0.6876	0.7542
tSC (hr)	78.51 ± 6.46	N/A	76.28 ± 9.06	76.28 ± 9.06	N/A	0.5716	0.5716	N/A
tM (hr)	83.31 ± 5.68	81.52 ± 6.23	80.20 ± 7.45	80.40 ± 7.23	0.5962	0.2018	0.2174	0.6138
tSB (hr)	93.86 ± 5.60	92.95 ± 4.53	90.09 ± 5.32	90.55 ± 5.27	0.8868	0.1015	0.1478	0.1830
tB (hr)	102.37 ± 6.95	98.74 ± 4.88	97.95 ± 5.69	98.07 ± 5.53	0.3749	0.0494	0.0564	0.7071
tEB (hr)	118.12 ± 6.71	N/A	118.38 ± 2.93	118.38 ± 2.93	N/A	0.7773	0.7773	N/A
GDF- 9 (pg/mL)	6103.9 ± 4123.3	7557.7 ± 4989.8	6358.4 ± 3631.4	6540.9 ± 3826.9	0.6590	0.8148	0.7539	0.5207
Daily GDF-9 (pg/mL/day)	1220.8 ± 824.7	1511.6 ± 997.9	1271.7 ± 726.3	1308.2 ± 765.4	0.6590	0.8148	0.7539	0.5207

Mann-Whitney test. Values expressed as mean ± SD.

Table 5d. Time-lapse morphokinetics and GDF-9 measurements in spent medium of Day 6 embryos according to pregnancy outcome

DAY 6								
	No pregnancy (NP) (N=21)	Biochemical Pregnancies (BP)			p-values			
		Miscarriage (MC) (N=8)	Clinical (CP) (n=27)	Total BP (n=35)	NP vs MC	NP vs CP	NP vs Total BP	MC vs CP
tPNa (hr)	8.35 ± 1.88	7.41 ± 1.51	8.03 ± 2.04	7.89 ± 1.93	0.2831	0.4994	0.3563	0.4795
tPNf (hr)	23.14 ± 2.7	24.06 ± 3.55	25.48 ± 3.00	25.16 ± 3.14	0.7697	0.0091	0.0260	0.2233
t2 (hr)	25.64 ± 2.74	26.49 ± 3.73	27.88 ± 3.03	27.56 ± 3.20	0.7697	0.0109	0.0296	0.1573
t3 (hr)	36.08 ± 4.10	36.96 ± 2.25	39.91 ± 3.47	39.28 ± 3.44	0.5439	0.0024	0.0068	0.0782
t4 (hr)	37.56 ± 4.3	38.36 ± 2.50	41.41 ± 3.87	40.71 ± 3.80	0.6963	0.0055	0.0166	0.0648
t5 (hr)	48.26 ± 5.58	50.81 ± 4.22	54.41 ± 4.14	53.60 ± 4.36	0.2685	0.0004	0.0010	0.1190
t6 (hr)	51.04 ± 7.14	50.89 ± 6.48	56.12 ± 4.49	54.77 ± 5.48	0.6111	0.0038	0.0128	0.0579
t7 (hr)	54.19 ± 8.57	53.94 ± 4.49	58.28 ± 5.58	57.23 ± 5.59	0.6907	0.0141	0.0330	0.0745
t8 (hr)	55.84 ± 9.71	56.53 ± 6.96	62.50 ± 8.61	61.01 ± 8.54	0.9223	0.0033	0.0172	0.0502
tSC (hr)	79.39 ± 16.01	71.94 ± 12.0	83.31 ± 11.07	80.47 ± 11.91	0.4795	0.6434	0.9035	0.1655
tM (hr)	87.26 ± 7.60	90.40 ± 6.18	91.05 ± 8.36	90.90 ± 7.84	0.2416	0.0756	0.0639	0.4795
tSB (hr)	97.01 ± 6.75	100.29 ± 4.77	103.42 ± 6.28	102.83 ± 6.09	0.2382	0.0076	0.0093	0.3106
tB (hr)	106.55 ± 9.63	108.67 ± 3.84	111.42 ± 6.40	110.78 ± 5.96	0.1184	0.0041	0.0040	0.3101
tEB (hr)	135.55 ± 6.27	128.36 ± 6.34	136.01 ± 5.87	134.10 ± 6.66	0.2752	0.6439	1.0000	0.1160
GDF- 9 (pg/mL)	4110.4 ± 2638.9	5541.3 ± 3591.8	3319.8 ± 2333.7	3827.6 ± 2777.9	0.3539	0.3085	0.6116	0.1073
Daily GDF-9 (pg/mL/day)	685.1 ± 439.8	923.6 ± 598.6	553.3 ± 389.0	637.9 ± 463.08	0.3539	0.3085	0.6116	0.1073

Mann-Whitney test. Values expressed as mean ± SD.

When looking specifically at biochemical and clinical pregnancies, there were clear significant differences between Day 5 and Day 6 embryos in almost all the time points starting from syngamy (2PN fade). This is an expected outcome since the Day 6 embryos, by definition, blastulated one day later, but it is interesting to note that this delay in development occurred very early in culture. Since there were no significant difference in the time when the 2PN appeared, it would seem that a significantly lower duration for the 2PNs to fuse could be indicative of slower blastocyst development. However, this delay did not appear to hamper implantation potential as these embryos all resulted in a pregnancy, whether it be biochemical or clinical.



Table 5e. Time-lapse morphokinetics and GDF-9 measurements in spent medium of Day 5 versus Day 6 embryos according to pregnancy outcome

	Clinical Pregnancy			Total Biochemical (Clinical + Miscarriages)		
	Day 5 (n=39)	Day 6 (n=27)	p-value	Day 5 (n=46)	Day 6 (n=35)	p-value
tPNa (hr)	7.81 ± 2.19	8.03 ± 2.04	0.4805	7.74 ± 2.10	7.89 ± 1.93	0.5705
tPNf (hr)	22.65 ± 2.30	25.48 ± 3.00	0.0003	22.75 ± 2.36	25.16 ± 3.14	0.0009
t2 (hr)	25.03 ± 2.44	27.88 ± 3.03	0.0003	25.17 ± 2.54	27.56 ± 3.20	0.0011
t3 (hr)	35.27 ± 3.92	39.91 ± 3.47	<0.0001	35.52 ± 3.97	39.28 ± 3.44	0.0001
t4 (hr)	36.56 ± 3.24	41.41 ± 3.87	<0.0001	36.73 ± 3.40	40.71 ± 3.80	<0.0001
t5 (hr)	48.18 ± 4.57	54.41 ± 4.14	<0.0001	48.15 ± 4.99	53.60 ± 4.36	<0.0001
t6 (hr)	49.48 ± 4.87	56.12 ± 4.49	<0.0001	49.62 ± 4.88	54.77 ± 5.48	0.0001
t7 (hr)	51.19 ± 5.63	58.28 ± 5.58	<0.0001	51.27 ± 5.65	57.23 ± 5.59	<0.0001
t8 (hr)	53.28 ± 6.97	62.50 ± 8.61	<0.0001	53.11 ± 6.77	61.01 ± 8.54	<0.0001
tSC (hr)	76.28 ± 9.06	83.31 ± 11.07	0.1208	76.28 ± 9.06	80.47 ± 11.91	0.2914
tM (hr)	80.20 ± 7.45	91.05 ± 8.36	<0.0001	80.40 ± 7.23	90.90 ± 7.84	<0.0001
tSB (hr)	90.09 ± 5.32	103.42 ± 6.28	<0.0001	90.55 ± 5.27	102.83 ± 6.09	<0.0001
tB (hr)	97.95 ± 5.69	111.42 ± 6.40	<0.0001	98.07 ± 5.53	110.78 ± 5.96	<0.0001
tEB (hr)	118.38 ± 2.93	136.01 ± 5.87	0.0002	118.38 ± 2.93	134.10 ± 6.66	0.0001
GDF- 9 (pg/mL)	6358.44 ± 3631.40	3319.80 ± 2333.70	0.0002	6540.94 ± 3826.89	3827.56 ± 2777.88	0.0007
Daily GDF- 9 (pg/mL/day)	1271.69 ± 726.28	553.30 ± 388.95	<0.0001	1308.19 ± 765.38	637.93 ± 462.98	<0.0001

Mann-Whitney test. Values expressed as mean ± SD.

The mean patient age for this study is depicted in Table 5f. There was no significant difference in age between any of the pregnancy groups on Day 5 versus Day 6.

Table 5f. Mean patient age in each of the pregnancy groups using Day 5 versus Day 6 embryos for transfer

Pregnancy groups	Patient Age (yrs)		p-value
	Day 5	Day 6	
No pregnancy	35.6 ± 4.1 (n=11)	34.3 ± 4.8 (n=21)	0.3115
Biochemical	34.9 ± 4.4 (n=46)	34.2 ± 4.2 (n=35)	0.5868
• Clinical	34.3 ± 4.1 (n=39)	34.0 ± 4.2 (n=27)	0.7395
• Miscarriage	38.3 ± 5.0 (n=7)	35.2 ± 4.4 (n=8)	0.1321
TOTAL	35.1 ± 4.3 (n=57)	34.3 ± 4.4 (n=56)	0.3107

Mann-Whitney test. Values expressed as mean ± SD.

Due to the decrease in GDF-9 from Day 5 to Day 6, the question arose to when this decline starts. Since there is evidence that GDF-9 starts to decrease after the 8-cell stage, GDF-9 values in spent media on Day 5 of embryos that were good quality on Day 3 that arrested on Day 5 were compared to those that developed to blastocysts. Even though the sample size is very small and the data is preliminary, there was no significant difference in the GDF-9 values on Day 5 (3,950.4±2,207.0 pg/mL vs 4,273.4±1,293.5 pg/mL; P=0.7343) between these two groups (Table 5g). This would indicate that GDF-9 exists irrespective of whether the embryo develops into a blastocyst or remains at the 8-cell or

cleavage stage. This may suggest that after Day 3, GDF-9 production is halted or slowed down.

Table 5g. GDF-9 in Day 5 spent media of embryos that were good quality on Day 3 (8-cell) which arrested on Day 5 versus those that developed into expanded blastocysts.

	Arrested (n=10)	Expanded Blastocysts (n=7)	p-value
GDF- 9 (pg/mL)	3,950.4± 2,207.0	4,273.4± 1,293.5	P = 0.7343

Mann-Whitney test. Values expressed as mean ± SD.

4.6. Oxidation-reduction potential (ORP) in spent medium of good quality Day 3 embryos and their ability to reach the blastocyst stage.

Control spent medium on Day 6 without any embryos showed an overall ORP value of 220.6±21.3 mV, with a range of 178.2 mv to 261.9 mV. Comparing the corresponding control spent medium in the arrested group versus the blastocyst group, the ORP showed no significant differences were noted in the ORP (222.29±20.86 mV vs 219.12±21.77 mV; p=0.5423; Table 6a, Figure 16). This would illustrate that the differences seen in embryo development between these two groups are not related to culture but rather intrinsic factors inherent to the embryo itself.

The spent medium from blastocysts showed significantly higher levels of ORP (219.43±19.58 mV vs 209.78±13.88 mV; p=0.0172) than that of arrested embryos (Table

6a; Figure 17). Furthermore, the ORP of spent medium in which embryos developed into blastocysts showed no difference in ORP from the control medium as evidenced in Table 6a (219.43 ± 19.58 mV vs 219.12 ± 21.77 mV; $p=0.9469$). It would appear that since these embryos are considered healthy and had normal embryo development to the blastocyst stage, that they are able to maintain the oxidative reductive balance. On the contrary, spent medium from arrested embryos showed a significant decrease in ORP from the control medium (209.78 ± 13.88 mV vs 222.29 ± 20.86 mV; $p=0.0045$; Table 6a). This would indicate either an uptake of oxidants from the media or a release of antioxidants into the media. The latter seems more likely as these arrested embryos start to degenerate and cell lysis occurs, various factors are released which could shift the ORP balance towards the reduction side.

Table 6a. Oxidation-reduction potential (ORP) of spent medium of embryos that were good quality on Day 3 that either arrested or developed into blastocysts on Day 6.

Culture media	Arrested (n=35)	Expanded Blastocysts (n=41)	p-value
ORP Control (mV)	222.29 ± 20.86	219.12 ± 21.77	$p=0.5423$
ORP Embryo (mV)	209.78 ± 13.88	219.43 ± 19.58	$p=0.0172$
p-value	$p=0.0045$	$p=0.9469$	

Mann-Whitney test. Values expressed as mean \pm SD.

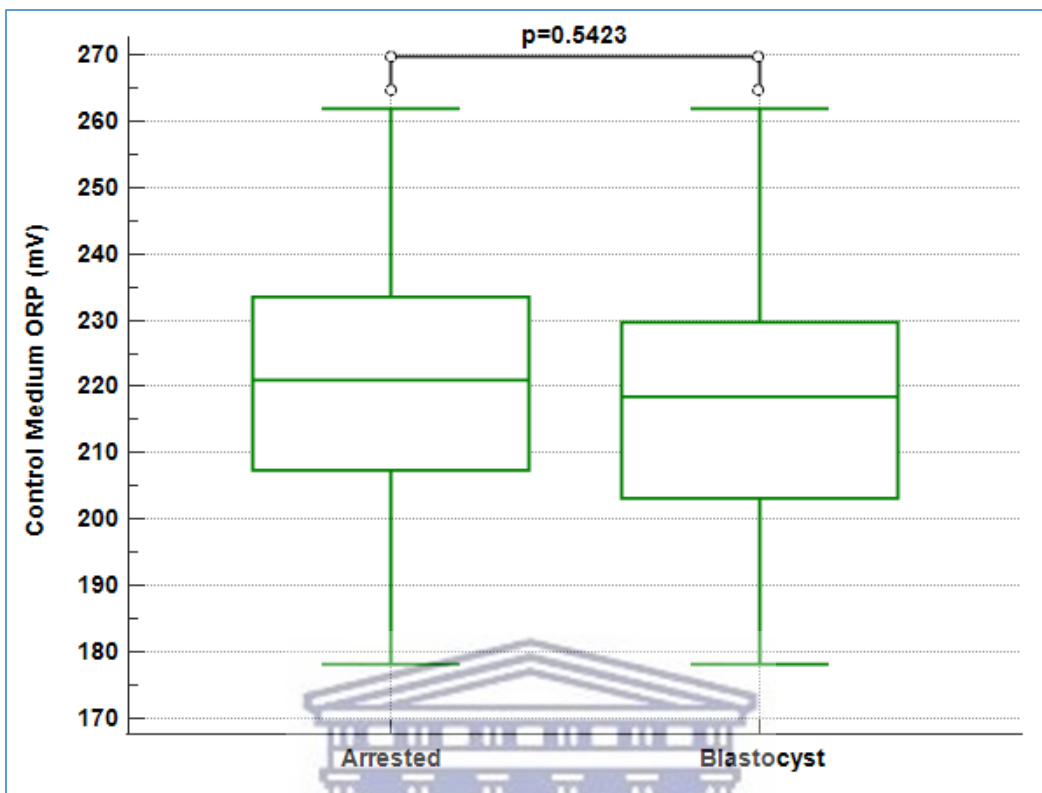


Figure 16. Oxidation-reduction potential (ORP) of control spent medium in the arrested versus blastocyst group showing no significant difference ($p=0.5423$) indicating that the oxidative stress of the medium did not contribute the arrested development.

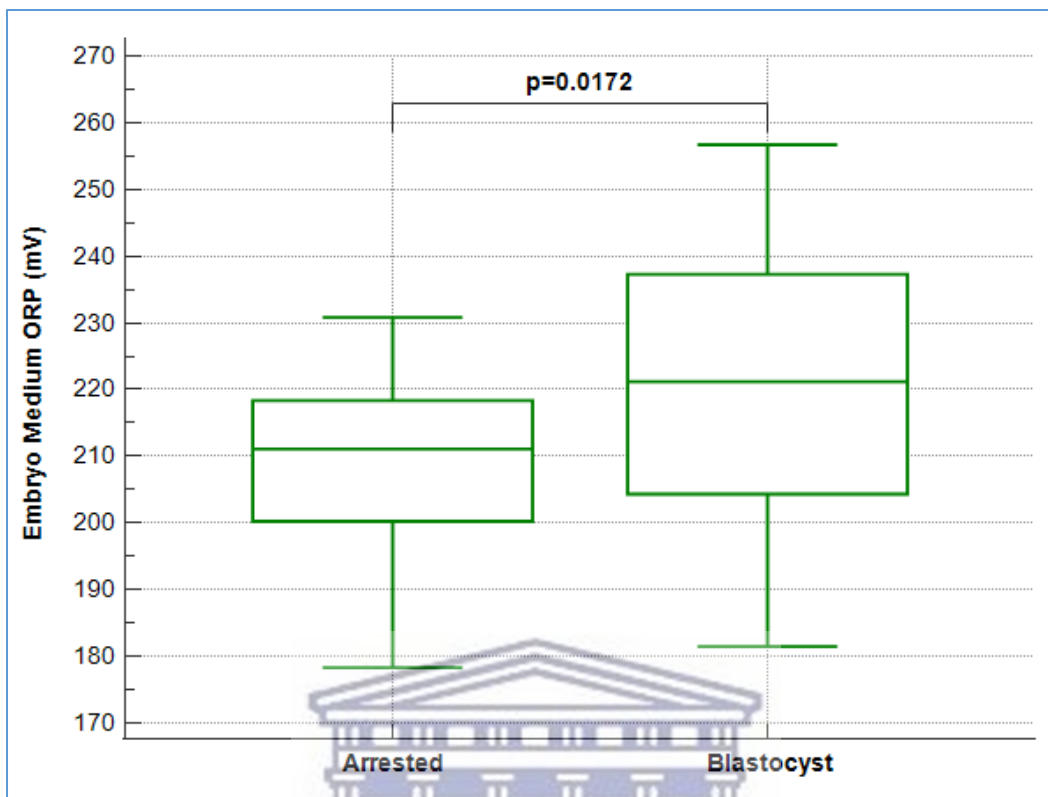


Figure 17. Oxidation-reduction potential (ORP) of embryo spent medium in the arrested versus blastocyst group showing significantly higher levels of ORP in the blastocyst group ($p=0.0172$).

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With regards to TLM, the only differences noted between the arrested embryos and blastocysts were at the time of pronuclear fusion (24.72 ± 3.42 hr vs 23.22 ± 2.68 hr; $p=0.03$), and the times for morula (97.41 ± 11.46 hr vs 91.59 ± 7.19 hr; $p=0.0121$), start of blastulation (110.69 ± 11.53 hr vs 102.78 ± 7.41 hr; $p=0.0040$) and blastocyst (120.04 ± 9.42 hr vs 113.08 ± 8.40 hr; $p=0.0105$) as noted in Table 6b. Even though some of the arrested embryos did start to blastulate at 110.69 ± 11.53 hr, and some developed into blastocysts at 120.04 ± 9.42 , none of them developed further into expanded blastocysts.

Table 6b. Time-lapse embryo morphokinetics of good quality Day 3 human embryos (8-cell) that arrested versus those that developed into expanded blastocysts.

Time points	Arrested (n=35)	Expanded Blastocysts (n=41)	p-value
tPN appear (hr)	8.74 ± 3.36	7.96 ± 1.20	0.3328
tPN fade (hr)	24.72 ± 3.42	23.22 ± 2.68	0.0300
t2 (hr)	27.13 ± 3.46	25.80 ± 2.76	0.0712
t3 (hr)	34.36 ± 6.01	35.98 ± 3.78	0.4889
t4 (hr)	38.11 ± 5.85	37.78 ± 4.71	0.2305
t5 (hr)	47.07 ± 7.57	48.60 ± 5.44	0.2105
t6 (hr)	49.57 ± 9.11	51.54 ± 4.95	0.1111
t7 (hr)	56.46 ± 8.76	55.40 ± 7.43	0.4129
t8 (hr)	61.56 ± 9.29	59.10 ± 8.78	0.1051
t9+ (hr)	72.29 ± 12.24	71.23 ± 7.06	0.8478
tSC (hr)	86.72 ± 11.63	84.30 ± 8.31	0.1796
tM (hr)	97.41 ± 11.46	91.59 ± 7.19	0.0121
tSB (hr)	110.69 ± 11.53	102.78 ± 7.41	0.0040
tB (hr)	120.04 ± 9.42	113.08 ± 8.40	0.0105
tEB (hr)	N/A	130.09 ± 7.77	N/A
tHB (hr)	N/A	134.04 ± 6.43	N/A

Mann-Whitney test. Values expressed as mean ± SD.

5. DISCUSSION

This thesis investigated various aspects of assisted reproductive technologies to improve the methodologies of embryo culture to yield more high quality embryos (blastocysts) available for embryo transfer and also to find certain markers in spent culture media which could be predictive of implantation potential. This was achieved through a series of experiments, first to establish the best culture medium and incubation system using human embryos, followed by evaluating various culture medium supplements such as insulin and IGF-1, and reducing the oxygen tension during the incubation period to more physiological levels with mouse embryos. Since these studies employed mouse embryos, two different strains of mice were compared to determine their suitability in quality control assays and future research studies.

After extended culture of human embryos until Day 5 and 6, the remaining spent medium was collected and analyzed for the presence of GDF-9 and oxidative stress to evaluate if these markers have any prognostic value in predicting which embryos would be more likely to achieve a pregnancy.

5.1. Comparison of human blastocyst development using sequential medium versus One-Step™ medium in EmbryoScope™ and Planer incubators

An optimum culture system should provide for a stable environment that mimics the *in vivo* conditions as much as possible, in terms of temperature, gas concentrations, osmolarity and pH (Pool et al. 2012). Several different culture media are available for

embryo development. Sequential media are specifically catered to the nutritional requirements of the embryo depending on the stage of development (Gardner et al. 2002), whereas single system culture media systems provide all nutrients and allow the embryos to utilize these nutrients as it needs (Biggers and Summers 2008). Reports have shown no differences in the choice of the culture system as both can support embryo development (Youssef et al. 2015, Cairo Consensus Group 2020)

The advantage of using a single step medium in conjunction with the EmbryoScope™ is that it minimizes stress to the embryo which could result from pH changes and fluctuations in temperature. The one concern of using a single step medium is to reduce the buildup of ammonia, but this can be prevented by using a stable form of glutamine (Macklon et al. 2002, Biggers and Summers 2008). It was originally suggested that ammonia buildup may cause detrimental effects on mouse blastocyst development (Lane and Gardner 2003) but this was later disputed and found to be unjustified (Biggers et al. 2004). Moreover, it was shown that the ammonium concentrations were lower in single step media (Hardarson et al. 2015).

Numerous studies have evaluated various culture medium compositions and their ability to yield good embryo development (Van Langendonck et al. 2001, Aoki et al. 2005, Xella et al. 2010). There is a wide variety of components used in different culture media available on the market, with pyruvate, lactate and amino acid concentrations being the most different (Morbeck et al. 2014). Sequential media are based on the fact that during early embryo development (Gardner and Lane 1998), only a few amino acids are needed,

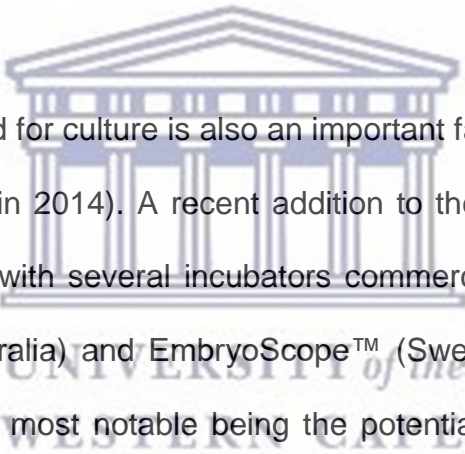
but in the compaction phase, all 20 amino acids are required (Gardner et al. 2000). The main benefit to extend culture to Day 5 or 6 to the blastocyst stage is that it eliminates those embryos with limited or no developmental potential (Gardner and Lane 1998). Single step media have been reported to have higher fertilization rate, significantly higher percentage of good quality blastocyst and implantation rates compared to sequential media (Wirleitner et al. 2010).

In this study, we compared the EmbryoScope™ time-lapse incubator with the Planer benchtop incubator with two different culture media systems, notably sequential and One-Step™. With regards to culture media, there were no significant differences in fertilization rate, contrary to previous reports (Wirleitner et al. 2010). However, the total blastocyst development rate (Day 5 and Day 6) was significantly higher in embryos cultured in One-Step™ medium when compared to the sequential media. Furthermore, the individual Day 5 and Day 6 blastocyst rates were also higher in One-Step™ medium, but this difference was not significant. This would suggest that embryos tend to reach the blastocyst stage sooner on Day 5 in One-Step™ medium compared to sequential media.

The selection of culture media is of vital importance in the IVF lab in maintaining viable embryos. Negative reports have been published about the association of the type of culture medium and ectopic pregnancies (Lin et al. 2015). The ectopic pregnancy rate is 2.5-5 fold higher in IVF cycles than normal conception (Smith et al. 2013). Ectopic pregnancies account for 3-4% of pregnancy related deaths (Llaneza-Suarez et al. 2014). Therefore, the choice of the culture medium should be an important decision since its

consequences could have long-term implications far beyond the embryo development during the first six days of culture.

The importance of the culture medium extends beyond that of embryo development. Babies born from IVF have increased incidence of low birth weight (Schieve et al. 2002), the reason for which is unclear, due to factors such as infertility, increase incidence of multiple births and genetic background (Davies et al. 2012). Some have postulated that the composition of the culture medium may affect the health of the offspring (Dumoulin et al. 2010, Nelissen et al. 2012).



The incubation system used for culture is also an important factor contributing to optimal embryo development (Swain 2014). A recent addition to the embryo culture system is time-lapse morphokinetics with several incubators commercially available such as the Miri (Denmark), Geri (Australia) and EmbryoScope™ (Sweden). The EmbryoScope™ has many advantages, the most notable being the potential to select the most viable embryos to transfer and identifying timing differences between embryos with the same morphological classification (Wong et al. 2010, Conaghan 2014, Kirkegaard et al. 2015). Furthermore, it can help in observing a number of developmental anomalies which cannot be seen with traditional culture. Several studies have shown that direct cleavage, blastomere fusion, and reverse cleavages are negatively associated with embryo implantation (Desai et al. 2014). There are several reports demonstrating the predictive value of TLM (Pribenszky et al. 2010, Meseguer et al. 2012, Campbell et al. 2013),

whereas other reports (Sfontouris et al. 2016, Dieamant et al. 2017) and a recent Cochrane review (Youssef et al. 2015) showed no benefit of TLM.

In this study, the EmbryoScope™ incubator showed significant improvement over the Planer incubator in terms of total blastocyst development rate (Day 5 and Day 6), irrespective of which culture system was used. However, when looking at culture systems *per se*, the blastocyst development was still higher in the EmbryoScope™ than the Planer, using either sequential or One-Step™ medium, but these differences were not statistically significant. Furthermore, the EmbryoScope™ yielded significantly more blastocysts on Day 5 demonstrating a shorter time to blastocyst, which could in turn could have a better potential for implantation. However, no significant differences in blastocyst rates were noted on Day 6, suggesting that the increase in total blastocyst development was solely due to increase in Day 5 blastocysts.

There appears to be no harm to the embryos with repeated light exposure during image acquisition every 10 min in the EmbryoScope™. Since the EmbryoScope™ uses red light at 625 nm, which is a much lower irradiation level compared to white light, it does not decrease embryo development or quality at the cleavage or blastocyst stage (Nakahara et al. 2010, Cruz et al. 2012, Sciorio et al. 2018) or clinical pregnancy rate (Mio and Maeda 2008).

The one variable in this study was that eggs/embryos were cultured in 25 µL medium in the EmbryoScope™ and in 30 µL of medium in the Planer incubators. It is unclear if this

5 μ L difference would have any impact on embryo quality. Another difference is that the EmbryoScope™ is not humidified, whereas the Planer is. Traditionally, culture incubators are typically humidified to prevent evaporation and subsequent increases in osmolarity (Lane et al. 2008, Swain et al. 2012). As most culture dishes are overlaid with mineral oil which slows down the evaporation process, humidity is not as critical for these dishes (Swain 2014).

Since the EmbryoScope™ incubator can support embryo development as well as the Planer benchtop incubator and yield more blastocysts on Day 5, the added advantages of reducing door openings, media changes and morphokinetics data make it a suitable choice as an incubation system. Furthermore, to enhance these benefits of the EmbryoScope™, the One-Step™ medium, which resulted in improved blastocyst rates as compared to the sequential medium, should be the medium of choice, as it allows for the uninterrupted culture of embryos providing the most optimal environment.

5.2. Comparison of blastocyst development and time-lapse morphokinetics of mouse embryos cultured in One-Step™ medium supplemented with IGF-1 of insulin.

Various compounds have been added to culture media in attempts to promote embryo development. Exogenous insulin or IGF-1 have been shown to improve embryo development, as well as increasing cell proliferation (Harvey and Kaye 1990, Gardner and Kaye 1991) as well as reducing apoptosis (Behr and Wang 2004).

In this study, the effects of supplementing One-Step™ culture medium with and without either insulin or IGF-1 on blastocyst development and morphokinetics using two different mouse strains were compared. In both, the hybrid and C57 mouse models, IGF-1 showed no difference in either TLM or blastocyst development. However, embryos cultured in medium supplemented with insulin showed slower time to start of blastulation in the hybrid model. This was accompanied with a lower percentage of embryos reaching the blastocyst and expanded blastocyst stages. A similar trend was seen using the C57 mouse model where no differences were noted in TLM and blastocyst rate for IGF-1 cultured embryos, but embryo culture in the presence of insulin showed slower times to reach the morula and blastocyst stages. In addition, these embryos also showed significant reduction in blastocyst rate.

These findings are in contrast to those found in the literature. One of the concerns may be directed towards the concentration of insulin or IGF-1 used, since supraphysiological levels of IGF-1 have been shown to be embryotoxic in mice (Katagiri et al. 1996, Katagiri et al. 1997, Chi et al. 2000, Green and Day 2013, Irani et al. 2018). This negative effect is thought to be due to downregulation of IGF-1 receptors, thereby reducing the insulin-stimulated glucose uptake resulting in higher apoptosis (Chi et al. 2000, Irani et al. 2018). The 100 ng/mL concentration of insulin and IGF-1 used in this study was based on previous reports showing that 10-100 ng/mL IGF-1 stimulated nuclear maturation of immature oocytes whereas 1,000 ng/mL did not have any effect (Sirotkin et al. 1998). With regards to insulin, reports have shown improved clinical pregnancy rate and embryo development using concentrations as low as 50 ng/mL in humans (Fawzy et al. 2017).

Furthermore, it has also been shown that insulin concentrations of up to 10,000 $\mu\text{g}/\text{mL}$ showed no deleterious effects on embryo development in rats (Travers et al. 1989, Travers et al. 1992)

Another confounding factor in this study may be attributed to individual culture of embryos. It has been shown that when culturing embryos in groups of 3 or 4 per 20 μL droplet yielded significant improvement in blastocyst formation rate and subsequent clinical pregnancy rate (Fawzy et al. 2017). Other reports have shown that culturing up to 10-15 embryos in a drop yielded higher blastocyst rates (Green and Day 2013), possibly due to growth factors exuded by the embryos (O'Neill 1998).

5.3. Comparison of blastocyst development and time-lapse morphokinetics of mouse embryos cultured in a low oxygen (2%) environment.

During the first successful culture of a human embryo in 1971, Steptoe and Edwards described using a gas phase of 5% O_2 , along with 5% CO_2 and 90% nitrogen, instead of 20% atmospheric oxygen (Steptoe et al. 1971). Their decision was based on reports showing that the oxygen tension in the mammalian female reproductive tract was in the 2-8% range (Bishop 1957, Mastroianni and Jones 1965), with improved embryo culture of embryos using physiological O_2 (Whitten, 1969).

For many years, most embryology laboratories were still using atmospheric oxygen, possibly due increased costs of low oxygen systems, such as nitrogen gas, specialized incubators and oxygen sensors (Bontekoe et al. 2012, Morin 2017). However, due to

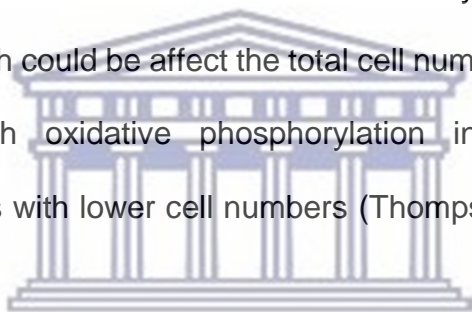
increasing evidence of benefit of low oxygen culture (Bontekoe et al. 2012, Nastri et al. 2016) and the availability of tri-gas incubators, most laboratories have made the switch from atmospheric oxygen to 5% oxygen. This resulted in improvements in embryo development and pregnancy rates.

Recently, due to the improved success of using low oxygen (5%), further efforts have focused on even ultra-low oxygen (2%) culture. It has been shown that the oxygen tension in the uterus is closer to 2% than 5% O₂ (Yedwab et al. 1976, Ottosen et al. 2006). It has been reported that 2% O₂ compared to 5% O₂ yielded higher rates of blastulation with fewer embryos that arrested at the cleavage stage (Kaser et al. 2018), while others showed no improvement (De Munck et al. 2019).

In this study, the development of 1-cell mouse embryos from Day 1 to Day 6, in terms of blastocyst development and TLM, using an ultra-low oxygen (2%) culture system was compared to a low oxygen (5%) system. In the hybrid mouse strain, slower embryo development was noted in all cell stages after the 8-cell stage. In the C57 strain, only the time to morula and expanded blastocyst stages were slower in the 2% O₂ group. Furthermore, the blastocyst development rate in this strain were significantly lower in 2% O₂ compared to 6% oxygen in the C57 strain, but no such differences were noted in the hybrid strain.

The embryo development rates obtained in this study are supported by others, who also showed no improved in embryo development, quality and utilization rate (De Munck et al.

2019), but are also in contrast to other previously published reports which did show improvements in embryo development (Kaser et al. 2018, Morin 2018). Even though the blastocyst development was not significantly different between the 2% O₂ and 6% O₂ groups, blastocysts resulting from the 2% O₂ culture had significantly fewer cells than the 6% O₂ blastocysts. This has been documented previously (Yang et al. 2016, Kaser et al. 2018), but the rationale for this remains unclear. Cleavage-stage embryos utilize oxidative phosphorylation for energy production, after which there is a shift towards glycolysis, where glucose becomes the primary energy substrate after blastulation (Gott et al. 1990). It has been postulated that the ultra-low O₂ environment may further promote this shift in metabolic preference, which could affect the total cell number. In addition, when using media supplemented with oxidative phosphorylation inhibitors, bovine embryos developed in to blastocysts with lower cell numbers (Thompson et al. 2000, Kaser et al. 2018).

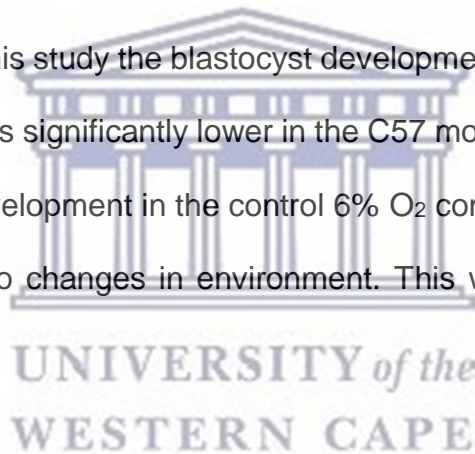


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It seems that the effect of oxygen could be dependent on the stage of development, since there is evidence that the oxygen tension in the reproductive tract changes from 8% O₂ in the fallopian tube, where fertilization occurs, to 2% in the uterus, where the embryo is at a blastocyst stage prior to implantations. For this reason, many studies have designed their experiments to reducing the oxygen tension to 2% only after Day 3 (Kaser et al. 2018, Morin 2018, De Munck et al. 2019). This study cultured embryos continuously in 2% oxygen from Day 1, which does not show any deleterious effect from Day 1 to Day 3, but clearly retards development after Day 3. It is possible that the initial stages of embryogenesis may require more oxygen, as this is more physiologically in accordance

to that of the reproductive tract. The shift in oxygen after the 8-cell stage also matches the metabolic requirements of the embryo (Chason et al. 2011). From Day 0 to Day 3, the embryos produce energy through oxidative phosphorylation (Leese 1995), with very little oxygen consumption and biosynthetic activity (Morin 2017). However, after compaction, the growth rate of embryos increases significantly as well and oxygen consumption (Morin 2017). It has been suggested that in order to protect the embryo from an excessive levels of reactive oxygen species, it would be beneficial to reduce the oxygen concentration during this period of high metabolic activity (Guerin et al. 2001).

Interesting to note that in this study the blastocyst development was unaffected by 2% O₂ in the hybrid model, but was significantly lower in the C57 model. The C57 strain showed even slower blastocyst development in the control 6% O₂ compared to the hybrid model, making it more sensitive to changes in environment. This will be discussed in greater detail in next section.



This is the first study evaluating time-lapse morphokinetic of embryo development using 2% oxygen culture conditions. Previous studies have only focused on blastocyst development rate, embryo quality and cell numbers (Kaser et al. 2018, Morin 2018, De Munck et al. 2019). Even though the blastocyst development rate in the hybrid mouse strain showed no significant difference, TLM showed very clearly that culture in 2% oxygen retarded embryo development significantly after the 8-cell stage. Since it has been suggested that oxygen tension in the reproductive tract changes around the time of compaction, it appears that this is a crucial time point, which has also been reflected in

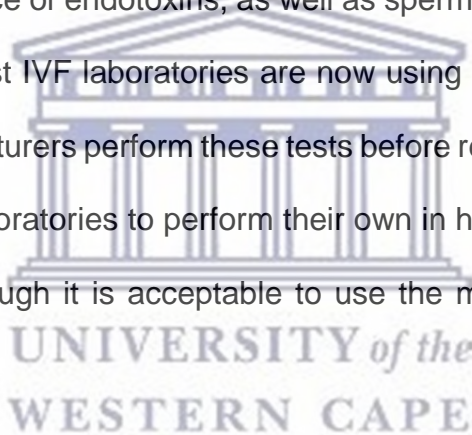
changes in metabolic requirements of the embryo. This slower compaction was also noted in the C57 strain when cultured in 2% O₂. Time-lapse morphokinetics offers very vital information in the mechanics of embryo development, which otherwise would be missed.

Future studies should be directed to more sequential approach by first culturing in 5% O₂ with subsequent reduction to 2% O₂ after Day 3, as opposed to the monophasic approach used in this study. Almost all the culture environment systems using 5-6% O₂ do so using a monophasic approach, even though culture media systems may use a sequential system to provide nutrients appropriate to different cell stages. From the data obtained in this study, even though embryo development up to the 8-cell stage was unaffected by 2% O₂, there may be some underlying detrimental effects that may only manifest after Day 3. The exact time of development when the switch from 5% O₂ to 2% O₂ takes place is still not clearly defined as most studies are reporting around the time of compaction as the embryo enters the uterus. Since it has been shown that ambient oxygen delays embryo development beginning with the 3rd cell cycle which is less than reported for mouse embryos (Wale and Gardner 2010), it is possible that delays in embryo development may be due to inappropriate oxygen concentration. Studies investigating the effects of low oxygen place embryos from a 5% O₂ incubator directly to a 2% O₂ environment. Even though they have shown improved blastocyst rates, an incubation system that can be programmed to gradually decrease the oxygen concentration from 5% to 2% O₂, starting from 8-cell stage to start of compaction may be more physiological and in conjunction

with time-lapse morphokinetic studies, may provide valuable insight to the dynamics of embryo development and culture.

5.4. Evaluation of a different strain of mouse embryos as a better model for research and quality control.

In order to use products for human embryo culture, it is imperative that the media or products are not embryotoxic and provide the best environment for embryo growth and development. Various assays exist to test the suitability of these media such as testing the pH, osmolarity, presence of endotoxins, as well as sperm survival assays and mouse embryo assays. Since most IVF laboratories are now using commercially produced IVF culture media, the manufacturers perform these tests before releasing them to the market. It is still prudent on IVF laboratories to perform their own in house testing, at least for pH as the minimum, even though it is acceptable to use the manufacturer's Certificate of Analysis.



The mouse embryo assay (MEA) is the standard assay used for QC with the endpoint being blastocyst development, but manufacturers do not report the strain of mouse embryos used or the culture conditions of the assay. There has been criticism of the MEA in its lack of standardization in the number and type of embryos used, as well as the ability of mouse embryos to accurately represent human embryos (Chronopoulou and Harper 2015, Ainsworth et al. 2017). Therefore, the question arose as to whether all these mouse embryo assays are alike and what could influence the outcome of these assays. There have been reports where mineral oil which passed manufacturer's testing, was deemed

to be the source of embryotoxicity and this was further substantiated by the IVF laboratory's own independent in-house testing using a different strain of mice (Morbeck 2012). It became clear that some strains of mouse embryos are hardier than others and cannot detect subtle changes or presence of toxins which would otherwise be detrimental to human embryos. Recent additions to the mouse embryo assay include time-lapse morphokinetics (Wolff et al. 2013) which has increased sensitivity by incorporating multiple quantitative measures as opposed to traditional MEA which uses a qualitative endpoint of blastocyst development (Ainsworth et al. 2017).

Unpublished data from our IVF lab has shown that using 2-cell mouse embryos almost always had above 95% blastocyst formation rate. In order to have more stringent mouse embryo assays, 1-cell embryos were used instead of 2-cell embryos yielding similarly high blastocyst development rates, which prompted investigation into the strain of mouse embryo. This study compared the blastocyst development rates and morphokinetics of two different strains of mouse embryos in various experimental models.

Under normal IVF culture conditions, the C57 strain of mouse embryos showed slower development to each of the various cell stage time points than the hybrid model, which is commonly used in mouse quality control assays. Furthermore, fewer embryos from this strain reached the blastocyst stage than the hybrid strain, indicating that it may be more sensitive and difficult to culture even under normal conditions. When using this strain in experimental studies comparing various environmental conditions or media supplementations, the C57 strain continued to show slower time-lapse morphokinetics for

each of the time points assessed. In each of these experiments, this strain exhibited lower blastocyst development in each of the experimental models tested, compared to the hybrid strain. In fact, in one of the experiments evaluating the effects of ultra-low oxygen on embryo development, the C57 strain was significantly affected in terms of blastocyst development, whereas the hybrid model was not (Table 3d).

It is clear from the data obtained in this study, that there are difference in mouse strains in terms of their sensitivity that would affect the outcome of quality control assays. The main reason why mouse assays are used, or any animal model, is to mimic the human embryo culture system as much as possible. However, mouse embryos can develop into blastocysts using culture media made with tap water containing appreciable amounts of endotoxin (Fleetham et al. 1993), whereas human embryos have been shown to be sensitive to very small amounts of endotoxin as little as 1ng/mL (Fishel et al. 1988). Since human oocytes and embryos are far more sensitive to outside stimuli (Fishel et al. 1988) than mouse embryos, it is imperative to identify a strain of mouse embryos that closely match the sensitivity of human embryos. If blastocyst development rates are to be the end point for QC assays, then this study has shown that the hybrid strain that is most commonly used, is not a suitable model for these assays, since 97% of them reach the blastocyst stage (range=94.3% to 99.1%), irrespective of which culture conditions were used (Table 4e). In contrast, the overall blastocyst rate of C57 mouse embryos averaged 73% with a range of 62.5% to 81.4%, for all conditions tested.

Hybrid strains that are commonly used, mostly since they are not susceptible to the 2-cell block, respond well to superovulation, and produce embryos that have consistently high blastocyst development potential (Suzuki et al. 1996, Khan et al. 2013). It has been reported that outbred mice are more sensitive to F1 hybrid mice (Gardner et al. 2005) and inbred mice (Khan et al. 2013). The exact reason for the increased sensitivity remains unclear since it could be assumed that the increased genetic diversity in the outbred mice should result in increased reproductive vigor compared to inbred strains (Khan et al. 2013). Another hypothesis is that the reproductive vigor during inbreeding was higher since those that did not reproduce well were excluded from the development of the strain (Khan et al. 2013). The strains used in this study were an inbred strain (C57BL) and a hybrid strain, which was a cross from 2 hybrids, each of which had C57BL as the parent. According to the company's information, "hybrid mice are created using two inbred strains, resulting in offspring that are more resistant to sickness, have increased survival rate under stress, live longer and have larger litters than the parental strains" (<https://www.criver.com/sites/default/files/resources/HybridMiceDatasheet.pdf>). This hybrid strain is the most common strain used in QC testing of materials used in the lab as well as by manufacturers before releasing culture media products to the market. However, the guidelines for the MEA identifies a threshold of 80% blastocyst development in order to pass testing. In this study, this hybrid strain showed exceptional blastocyst development well above the 80% requirement. Since this hybrid was created specifically to be more resistant to stress and have high survival, it is designed to overcome any toxins or aberrant environmental conditions, making it a poor choice for assay

development. However, the parental inbred strain appears to be more sensitive, even under normal culture conditions, making it more susceptible to suboptimal environments.

Since it is clear from this study that different mouse strains can impact the outcome of the MEA, a careful consideration should be taken in selecting a mouse strain for QC testing. This is becoming increasingly important especially since major advancements on culture media systems and their supplements allow for better embryo development, and could make it difficult to detect minute traces of toxins in the media. Albumin, for instance, has been shown to chelate toxins in the media and mask their effect (Morbeck et al. 2014, Mestres et al. 2019). On the other hand, protein supplements with human serum albumin (HSA) have been shown to protect embryos from toxic peroxides in mineral oil (Otsuki et al. 2007, Morbeck et al. 2012, Mestres et al. 2019). Another factor that can reduce the sensitivity of the MEA is group culture since it may improve IVF outcome, either through diffusion of unknown paracrine factors or maintenance of a stable microenvironment (Ainsworth et al. 2017). Other enhancements to the MEA to increase sensitivity include increasing the culture duration to 120-144 hr, total cell counts and morphokinetics (Ainsworth et al. 2017, Mestres et al. 2019)

Although it is usually desirable to have a high blastocyst rate in embryo culture, achieving this by using a genetically modified mouse strain with known enhancements in survivability defeats the purpose of having a QC program. QC testing should not only focus on having good blastocyst development as its end point but also have a mouse

strain that would make it more challenging to achieve those rates, by improving our culture conditions and detecting any toxins or detrimental conditions in the media.

5.5. GDF-9 levels in human embryo spent media and pregnancy outcome.

In a constant search for markers that can provide more information about the overall health of the embryo and its potential to implant and result in a healthy baby, there have been numerous factors that have been investigated such as traditional embryo quality grading, time-lapse morphokinetics, preimplantation genetic testing and mitochondrial integrity. Recent interest has shifted to metabolomics and spent culture media, in terms of proteins and other factors. Growth differentiation factor 9, along with bone morphogenetic protein 15 have been shown to play major roles in folliculogenesis and oogenesis and are important regulators of oocyte maturation (McNatty et al. 2005, Peng et al. 2013, Mottershead et al. 2015, Convissar et al. 2017).

There is an abundance of information with regards to GDF-9 in the ovary, follicular fluid and cumulus cells. Increased GDF-9 mRNA levels in cumulus cells has been shown to correlate with oocyte maturation, fertilization and embryo quality (Li et al. 2014). Moreover, adding exogenous GDF-9 to the *in-vitro* maturation medium resulted in improved oocyte maturation and improved embryo development (Yeo et al. 2008, Chatroudi et al. 2019). Absence of GDF-9 causes sterility due to a block at the primary stage of folliculogenesis (Dong et al. 1996). Other studies have shown that aberrant expressions of GDF-9 may be associated with polycystic ovarian syndrome and may also cause premature ovarian failure (Simpson et al. 2014). However, there is little information

about the presence of GDF-9 after fertilization and its expression in subsequent embryo development. No work has been published regarding the GDF-9 secretion of the human oocyte after ovulation or fertilization.

In this study, the assay was sensitive enough to measure GDF-9 in as little as 22 μ L spent medium from human embryos. Since no GDF-9 could be detected in the control medium (without embryos), the data shows that the GDF-9 measured by this assay originated from the embryo itself. This assay provides an easy, reliable method for measuring GDF-9 in small volume. GDF-9 levels in spent media of embryos on Day 5 and Day 6 that had subsequent frozen embryo transfers and compared the pregnancy rates were measured. There was a significant increase in biochemical and clinical pregnancy rates in Day 5 embryos compared to Day 6 embryos. This was accompanied with a significantly higher level of GDF-9 in Day 5 spent medium compared to Day 6, which is in agreement with the work shown by Liu et al. in the water buffalo (Liu et al. 2019), in which they showed a decrease in GDF-9 starting after the 8-cell stage.

Day 5 blastocysts vs Day 6 blastocysts showed higher biochemical pregnancy rate, clinical pregnancy rate and GDF-9 values in spent media. However, these Day 5 blastocysts showed no significant differences in time-lapse morphokinetic (TLM) parameters between the non-pregnant, clinically pregnant and early miscarriage groups. In contrast, when transferring Day 6 blastocysts, the non-pregnant group showed significant differences in TLM compared to the clinically pregnant group in all time points up to the 8-cell stage and start of blastulation. Interestingly, the clinical pregnancy groups

showed slower time-lapse embryo development than the non-pregnant group. This could indicate that cell divisions are time-controlled events and that embryos that develop faster are not necessarily indicative of a good embryo.

The lower daily GDF-9 values in Day 6 embryos could explain slower embryo development. The decline in GDF-9 observed on Day 6 could also indicate that embryo GDF-9 production declines after a certain point. In the water buffalo, GDF-9 has been shown to increase until the 8-cell stage and declining in the morula and blastocyst stages (Liu et al. 2019). The authors did not propose any rationale for this decline. Whether this decline in GDF-9 occurs in human embryos and when it happens is still unknown. In a study designed to measure GDF-9 levels in spent media on Day 5 from embryos that were good quality (8-cell) on Day 3, that either developed into expanded blastocysts versus those that arrested, preliminary data showed no significant difference in GDF-9. Even though the sample sizes are very small, and more studies need to be done, it suggests that the possibility that GDF-9 may not be produced by the embryo between the Day 3 and Day 5. Since GDF-9 is an oocyte secreted factor, it would seem plausible that after embryonic (zygotic) genomic activation on approximately Day 3, that the maternal contribution of GDF-9 is reduced or stopped, and that whatever GDF-9 has been produced remains in the spent medium and either starts to degrade or utilized by the embryo, which would explain the decline in GDF-9 from Day 5 to Day 6. Future studies can be directed to measure GDF-9 values on Day 3 with a changeover of media and tested again on Day 5 or Day 6. This would more accurately shed more light on the mechanics of GDF-9 in embryogenesis.

Another contributing factor to GDF-9 is age. It has been shown that with increased maternal age, expression of GDF-9 in follicular fluid decreases. It has been established that GDF-9 is essential for female fertility and folliculogenesis (Aaltonen et al. 1999, Juengel et al. 2004) and prevents granulosa cell apoptosis and follicular atresia (Orisaka et al. 2006). The absence of GDF-9 causes decreased granulosa cell proliferation, abnormal oocyte growth and follicular development failure (Hreinsson et al. 2002). It was shown that patients ≥ 35 years undergoing ovarian stimulation have a significantly lower GDF-9 concentration than those < 35 years (Han et al. 2011). This was associated with a corresponding lower number of oocytes retrieved and mature oocytes in the older group. However, their data showed no difference in clinical pregnancy rate between the two age groups. In this study, there were no significant differences in age between the pregnancy groups evaluated. Using the cut-off for age suggested by Han and co-workers (2011), data obtained in this study did not show any significant difference in GDF-9 concentration for patients < 35 and ≥ 35 years old.

GDF-9 did not show any significant differences between non-pregnant and pregnant groups of either Day 5 or Day 6 embryo transfers. From the limited sample size in this study, GDF-9 levels is not a suitable marker for predicting which embryos will result in implantation. Future studies might look into larger sample size to determine the importance of GDF-9 in embryos as it relates to pregnancy outcome, and also focus on the mechanisms by which the GDF-9 affects embryo development.

5.6. Oxidation-reduction potential (ORP) in spent media of good quality Day 3 human embryos and their ability to reach the blastocyst stage.

Reactive oxygen species have been associated with almost all aspects of reproductive function affecting ART outcome ranging from folliculogenesis, abnormal sperm parameters, sperm DNA fragmentation and abnormal embryo development (Agarwal and Saleh 2002, Agarwal et al. 2003, Agarwal and Allamaneni 2004, Pasqualotto et al. 2004, Sikka 2004, Wiener-Megnazi et al. 2004, Agarwal et al. 2006). ORP provides a comprehensive measure of oxidative stress by analyzing all the oxidants and antioxidants in a sample (Agarwal et al. 2016). Imbalances in ORP have been accurately associated with cellular damage resulting in the progression of acute traumatic injuries and chronic diseases (Shapiro 1972, Rael et al. 2007, Rael et al. 2009, Rael et al. 2009, Agarwal et al. 2016). The MiOXSYS® system has been shown to be a useful tool in reliably measuring ORP levels in semen and seminal plasma (Agarwal et al. 2016). The benefits of this device is that it is relatively cost effective, easy to use, and requires very little sample volume (Agarwal et al. 2016).

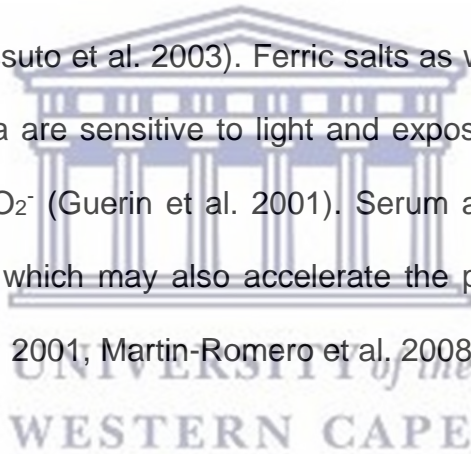
In this study, the data shows that good quality Day 3 embryos that reached the blastocyst stage had a higher ORP than those that did not. This is in contrast to the belief that higher oxidative stress causes poor embryo development. Further evaluation of the data showed no difference in ORP between the spent media of expanded blastocysts compared to control media with no embryos. This would indicate that the embryos that developed normally and reached the blastocyst stage were able to maintain its oxidative balance. In contrast, the arrested embryos showed significantly lower ORP levels in the spent

medium compared to their corresponding control medium. The lowered ORP would indicate a shift towards the antioxidant side. It appears that the oxidative stress measured in the medium was more a result of the metabolism of the embryo rather than having an effect on embryo development itself. Since there was no difference in ORP between spent medium of blastocysts compared to controls, one may assume that the healthy embryos are the ones that are able to maintain the balance between oxidants and antioxidants, during their development and cell divisions.

Furthermore, time-lapse morphokinetics showed significant differences in arrested embryos first at the syngamy stage, and then again from the morula, start of blastulation and blastocyst stages. Even though some of these embryos did reach the blastocyst stage, they were not fully expanded blastocysts. With the exception of the delay in pronuclear fusion, all time points leading up to the 8-cell stage are very similar. Only from the compaction stage onward, were the delays in the various time points very pronounced. This is typically at the time that genomic activation occurs, but since these embryos were from sibling oocytes, one can rule out sperm contributions as this would be constant for both arrested and blastocyst groups. The reason for why some embryos arrest and others continue to develop remains unclear.

Reactive oxygen species are found in physiological media such as follicular fluid, in the fallopian tubes and uterus, where various processes such as oocyte maturation, fertilization and embryo development take place (Guerin et al. 2001, Bedaiwy et al. 2002, Pasqualotto et al. 2004). These fluids have been shown to contain antioxidants, which can neutralize

the harmful effects of ROS generated *in vivo* (Guerin et al. 1995, Paszkowski et al. 1995, Campos Petean et al. 2008). However, *in vitro*, the culture medium is supplemented with serum or serum substitutes, albumin, vitamins, or buffers, which could also be a source of ROS (Martin-Romero et al. 2008). Since increased intracellular ROS may result in a negative impact on oocyte maturation and early embryo cleavage (Hu et al. 2001, Bedaiwy et al. 2004), there is concern that the culture medium may also present additional negative to embryo development due to its various components. It has been well documented that culture conditions could alter gene expression and imprinting (Rinaudo and Schultz 2004) and the culture medium composition could be associated with monozygotic twinning (Cassuto et al. 2003). Ferric salts as well as light sensitive flavins found in buffers and media are sensitive to light and exposure to atmospheric oxygen resulting in production of O_2^- (Guerin et al. 2001). Serum and serum substitutes have various oxidase activities, which may also accelerate the production of ROS in these culture media (Guerin et al. 2001, Martin-Romero et al. 2008)



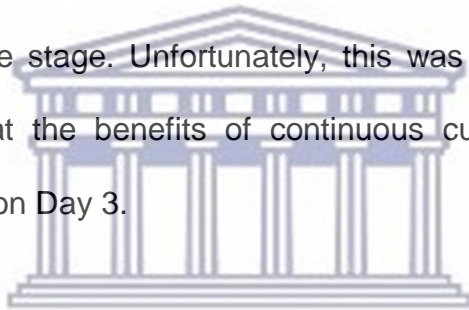
Commercially available culture media are usually supplemented with antioxidants to help protect against oxidative stress (Agarwal et al. 2006). Depending on the composition of the media, embryos produce ROS at different rates (Martin-Romero et al. 2008, Shih et al. 2014). Even though embryos have mechanisms to protect against ROS (Guyader-Joly et al. 1998), media supplements such as human serum albumin, help to scavenge ROS and protect against DNA damage (Ermilov et al. 1999). In mouse embryos, supplementing culture media with antioxidants such as acetyl-L-carnitine, N-acetyl-L-cysteine and α -lipoic acid have been shown to improve IVF outcome and subsequent

embryo development presumably by reducing oxidative stress (Truong and Gardner 2017). Embryos cultured with these antioxidants showed no improvement in fertilization rate or overall blastocyst formation rate but did significantly increase the cell numbers in both the trophectoderm as well as the inner cell mass. The same antioxidants, when added to vitrification and warming solutions increased cryosurvival and viability of mouse blastocysts after vitrification and warming (Truong and Gardner 2020). When applied to human IVF culture, these antioxidants achieved an increase in implantation rate and pregnancy rate for patients 35-40 years, presumably by reducing oxidative stress (Gardner et al. 2020). Other antioxidants, such as lycopene, have also been used to supplement culture media in the bovine model and were shown to significantly improve cleavage and blastocyst development rates as well as decreasing the intracellular ROS concentrations in oocytes and blastocysts (Chowdhury et al. 2018). In addition, embryos cultured in lycopene supplemented medium exhibited fewer apoptotic cells and increased cell numbers in the trophectoderm and inner cell mass (Chowdhury et al. 2018). Interesting to note from their study was that lycopene also up-regulated the expression of GDF-9, which has been shown to be correlated with both oocyte quality and embryo quality (Chowdhury et al. 2018).

Different IVF culture media exhibit different ORP values with Sage One-Step™ medium showing the least baseline ORP of 208.63 ± 4.77 mV compared to other commercial brands, ranging from 218.73 ± 2.01 mV to 273.83 ± 3.01 mV (Panner Selvam et al. 2018). In this study, the spent medium from control samples without any embryos had a baseline ORP of 220.6 ± 21.3 mV. The discrepancy in ORP between these two studies could be

due to the fact that the medium in this study was equilibrated for embryo culture conditions at 37°C at 5.5% CO₂ and 5% O₂ with a pH of 7.2, whereas the previous study evaluated ORP at room temperature with atmospheric oxygen with a pH of 7.6, which is suboptimal for embryo culture (Panner Selvam et al. 2018). pH plays a crucial role in the production of reactive oxygen species, with •SO₄⁻ radical being produced in acidic conditions whereas •OH and •O₂⁻ radicals are increased in intensity in basic pH conditions (Ahmad et al. 2015). The rate of ROS production has been shown to increase from pH of 6.0 up to a pH of 7.0, after which it declines sharply from pH of 7.0 to pH of 7.5 (Selivanov et al. 2008). These authors concluded that an increase in pH induces an increase in the generation of reactive oxygen species (Selivanov et al. 2008). With regards to temperature, several studies show that heat shock is closely related to the production of reactive oxygen species (Ealy et al. 1992, Malayer et al. 1992, Arechiga et al. 1995, Ealy et al. 1995). Furthermore, heat stress has been shown to increase the production of reactive oxygen species in the mouse oviduct and shift the redox state towards oxidation (Matsuzuka et al. 2005). Heat shock in early embryo development on Day 0 and Day 2 resulted in an increase in ROS whereas no increase in ROS was noted when heat stress was applied on Day 4 or Day 6 embryos (Sakatani et al. 2004). Early embryos (<8 cell) are more susceptible to heat stress and showed a high concentration of intracellular ROS (Sakatani et al. 2004). Embryos at the 8 cell stage are able to better withstand the high temperatures due to their ability to produce glutathione which also protects against oxidative stress (Gardiner and Reed 1995).

Furthermore, there was no difference in ORP between corresponding control (blank) spent media from the arrested embryo and blastocyst groups, illustrating that the medium was not a contributing or causative factor in the lack of development in these embryos. This is of particular importance since a common thought is that elevated ROS or oxidants would be detrimental to embryo development. On the contrary, it would appear that the rise in ORP presumably due to an increase in ROS could be a by-product of embryo development. This is especially evident in the blastocyst group where the embryo typically has over 100 cells and are more metabolically active than the arrested embryos. One limitation of this study was to measure the ORP of spent medium on Day 3 to evaluate a baseline from the cleavage stage. Unfortunately, this was not possible in our clinical setting, as it would defeat the benefits of continuous culture if the embryos were transferred to fresh media on Day 3.



A recent report did, however, look at the ROS levels of spent medium on Day 1, Day 3 and Day 5 and found no association with embryo development and quality (Lan et al. 2019). These authors also could not find any differences in ROS of spent media from normally fertilized zygotes and abnormal polyspermic zygotes or unfertilized oocytes. More importantly, there were no difference in ROS of spent medium on embryos from pregnant and non-pregnant groups. This is in contrast to other reports that have shown that ROS concentrations in spent culture media correlate with increased incidence of embryo fragmentation (Bedaiwy et al. 2004, Martin-Romero et al. 2008, Bedaiwy et al. 2010, Lee et al. 2012). In particular, high ROS concentration in Day 1 medium have been

associated with delayed embryo development, higher fragmentation rates and morphologically abnormal blastocysts (Bedaiwy et al. 2004).

A confounding factor in comparing data from different studies is the experimental design with regards to spent media with group culture or individual culture, the day at which the spent media was collected and the method for measuring oxidative stress, either directly as ROS or indirectly as ORP (which gives an overall picture of the balance of oxidants and antioxidants). Another variable is the culture environment, in that many of these studies were done at atmospheric oxygen, which can lead to an increase in oxidants, whereas this study used 5.0% O₂, which is common practice in many IVF laboratories today, due to the increased benefit of reducing oxidant levels. The minimal differences seen in the oxidative stress in this study could be due to the fact that the embryos were exposed to a reduced oxygen environment and the commercial culture media already being supplemented with some antioxidants, and both of these factors limit the oxidative stress on the embryo, making it easier for the embryo to maintain homeostasis and counteract the harmful effects of oxidative stress. Future research needs to focus on measuring ORP on a daily basis, to determine any changes over time, if any, correlates with embryo quality.

6. CONCLUSIONS

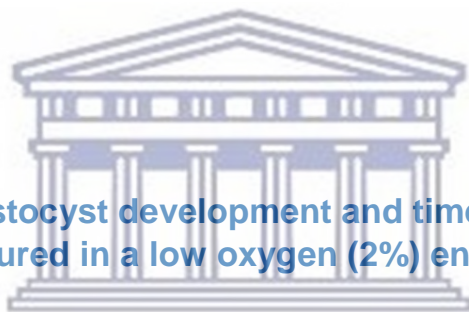
6.1. Comparison of human blastocyst development using sequential media versus One-Step™ medium in EmbryoScope™ and Planer incubators

One-Step™ media shows significantly improved total blastocyst development rate compared to sequential medium. One-Step™ medium can play a beneficial role in the IVF laboratory since it can (1) reduce dish preparation time, (2) eliminate the need for changing medium on Day 3, and (3) eliminate any disturbances to the embryo during culture until Day 5/6. Also, One-Step™ medium can maintain optimal culture conditions from fertilization to blastocyst in both EmbryoScope™ and Planer incubators.

The EmbryoScope™ incubator yielded higher total blastocyst rates than the Planer incubator, irrespective of which culture medium was used. Furthermore, a higher percentage of embryos reached the blastocyst stage in the EmbryoScope™ than the Planer incubator. The greatest advantage of the EmbryoScope™ is that embryos can be viewed without removing the embryos from the incubator, thereby minimizing any environmental stress, such as temperature and pH fluctuations. The data clearly shows that the combination of One-Step™ medium and culture in an EmbryoScope™ medium is superior to sequential media with Planer incubators, resulting in benefits not only physiologically but also from a practical and logical approach within a busy IVF laboratory.

6.2. Comparison of blastocyst development and time-lapse morphokinetics of mouse embryos cultured in One-Step™ medium supplemented with IGF-1 of insulin.

In hybrid embryos, insulin group had a slower time to start of blastulation (tSB) than control group and a lower blastocyst and expanded blastocyst rate. In C57 embryos, insulin group exhibited a slower time to morula (tM) and faster time to blastocyst (tB) than the controls with a corresponding lower rate of blastulation. IGF-1 showed no difference in TLM or blastocyst rates compared to control in both hybrid and C57 embryos. From these studies, there appears to be no benefit to adding insulin or IGF-1 to the culture media.



6.3. Comparison of blastocyst development and time-lapse morphokinetics of mouse embryos cultured in a low oxygen (2%) environment.

In ultra-low oxygen culture conditions, hybrid embryos showed no significant difference in time-lapse morphokinetics up to the 8 cell stage, but took a longer time for each time point after 8-cell stage. In contrast, the C57 embryos, when cultured in ultra-low O₂, were slower only in time to morula (tM) and expanded blastocyst (tEB). In addition, these embryos showed lower blastocyst and expanded blastocyst rates in the ultra-low O₂ group whereas the hybrid embryos did not.

6.4. Evaluation of a different strain of mouse embryos as a better model for research and quality control.

When comparing two mouse strains, the C57 strain had significant slower embryo development for all time points than hybrid embryos in all experimental groups (insulin, IGF-1 and ultra-low O₂). In addition, this strain also showed lower blastocyst and expanded blastocyst rates in all groups. This data clearly shows that the C57 mouse strain may be better to detect changes in media and culture conditions, and therefore be a better model for quality control assays in the lab and for research studies.

6.5. GDF-9 levels in human spent media and pregnancy outcome

Day 5 blastocysts showed higher biochemical pregnancy rate and clinical pregnancy rate as well as GDF-9 values in spent media compared to Day 6 blastocysts. Day 5 blastocysts showed no significant differences in time-lapse morphokinetic (TLM) parameters between the non-pregnant, clinically pregnant and early miscarriage groups. In Day 6 blastocysts, the non-pregnant group showed significant differences in TLM compared to the clinically pregnant group in all time points up to the 8-cell stage and start of blastulation. Interestingly, the clinical pregnancy groups showed slower time-lapse embryo development than the non-pregnant group.

The immunoassay was able to detect GDF-9 in as little as 22 µL of spent media. GDF-9 was undetectable in control media, showing that GDF-9 found in spent media originated from the embryo. GDF-9 did not show any significant differences between non-pregnant

and pregnant groups of Day 5 or Day 6 embryo transfers. Lower daily GDF-9 values in Day 6 embryos could explain slower embryo development. Decline in GDF-9 on Day 6 could also indicate that embryo GDF-9 production declines after a certain point. Due to small sample size of this study, more research needs to be done to understand the influence of GDF-9 on embryogenesis.

6.6. Oxidation-reduction potential (ORP) in spent media of good quality Day 3 human embryos and their ability to reach the blastocyst stage.

Spent medium from good quality Day 3 embryos that reached the blastocyst stage had a higher ORP than those that did not. However, ORP between spent medium from blastocysts compared to their corresponding control (blank) medium showed no significant differences, indicating that these embryos were able to maintain oxidative homeostasis. In contrast, spent medium from arrested embryos showed lower ORP than their corresponding controls, indicating an increase in reductive stress, which may possibly be due to apoptosis.

Furthermore, time-lapse morphokinetics showed significant differences in arrested embryos first at the syngamy stage, and then again from the morula, start of blastulation and blastocyst stages. Even though some these embryos did reach the blastocyst stage, they were not fully expanded blastocysts. With the exception of the delay in pronuclear fusion, all time points leading up to the 8-cell stage are very similar.

7. POSSIBLE FUTURE DEVELOPMENTS

The findings of this thesis has shed new light on various aspects of assisted reproductive technologies (ART) in ways to improved quality control (QC) assays to finding new markers for embryo quality and implantation potential.

From the mouse studies, it is very clear that the more sensitive C57 mouse strain of embryos have proven to be a better choice for QC testing of culture media and laboratory supplies than traditional hybrid embryos. In the future, many laboratories and manufacturers need to take this into account when developing new products for use in ART. The search for developing better a culture medium by means of adding different supplements is vital for the advancement of this field, and using this mouse strain would be beneficial before introducing any product to the market.

The data obtained in the GDF-9 study is very exciting and promising. Since GDF-9 was detectable in spent medium, additional studies need to be done to examine the dynamics of GDF-9 production throughout embryogenesis. This may provide more information about the physiology of growth factors and how it plays a role not only in oocyte maturation but also subsequent embryo development. Due to the small sample size in this study, additional work needs to be done to determine in GDF-9 in spent medium could be a non-invasive predictor for embryo health.

Similarly, oxidation-reduction potential (ORP) yielded interesting results and may elude to the fact that healthy embryos that reach the blastocyst stage have the capacity to

maintain oxidative homeostasis whereas poor quality embryos do not. This is an interesting finding and more studies need to be directed to ORP in the spent medium on Day 3 to provide more information about why some embryos arrest and others develop further. Ultimately, this data may also be compared to pregnancy outcome to help select better quality embryos for transfer in the future.

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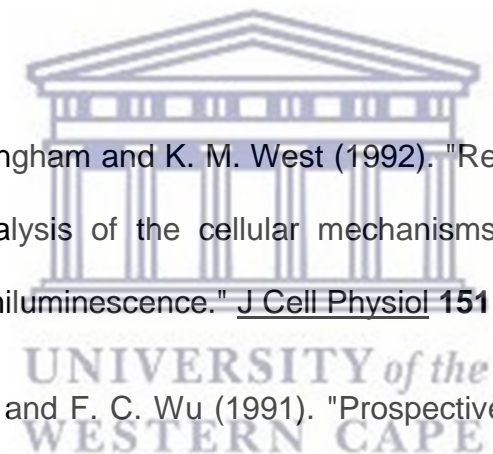
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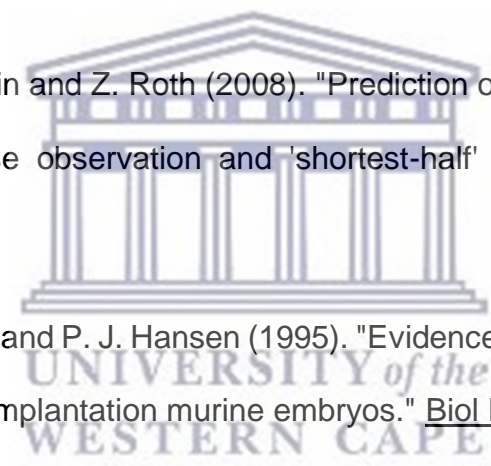
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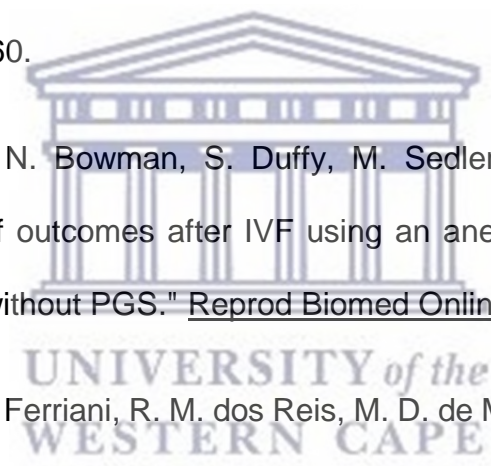
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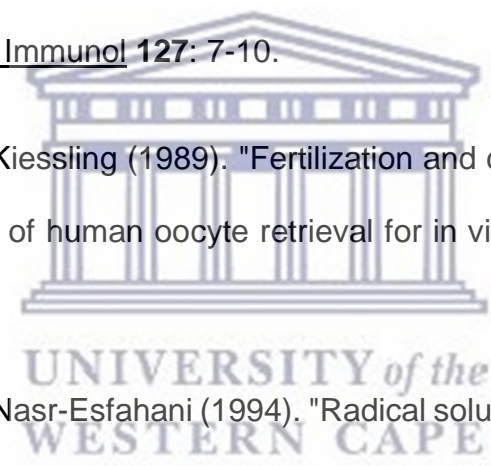
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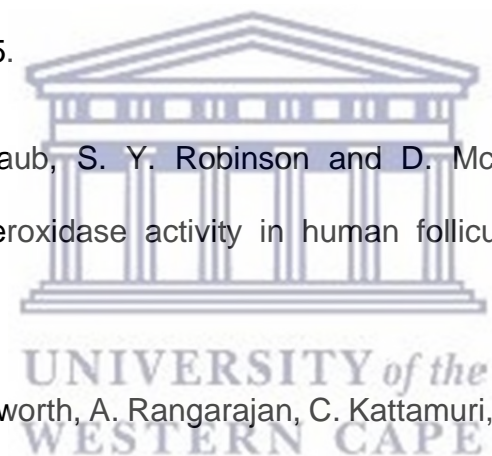
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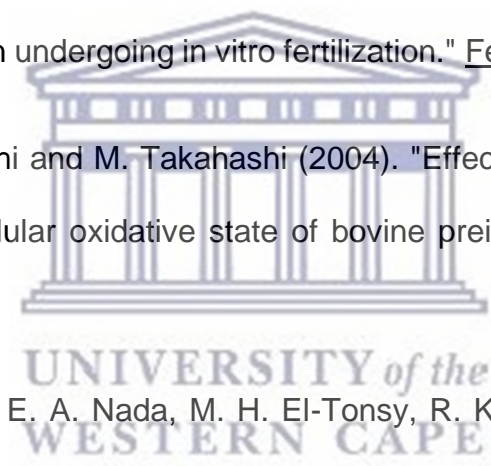
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