THE EFFECTS OF CO-CULTURING HUMAN EMBRYOS IN ONE-STEP CONTINUOUS CULTURE MEDIA ON BLASTULATION AND ASSISTED REPRODUCTIVE TECHNOLOGY OUTCOMES

By

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Background: The embryo culture system plays a vital role in optimizing human embryos' growth and development *in vitro*. At present, studies determining these conditions are often limited and contradictory, especially when considering the best culture media (sequential vs. continuous) and method of embryo culture (individual vs. co-culture) to use. As such, each laboratory is recommended to perform their own internal studies to determine which culture conditions give them the best clinical outcomes based upon their patient population and laboratory settings.

Aim: This study aims to investigate the effects of changing the embryo culture method from individually culturing embryos in Sequential Series™ embryo culture medium (ORIGIO®, Embryo Culture Method A) to co-culturing embryos in SAGE™ 1-Step™ with Human Albumin Solution (ORIGIO®, Embryo Culture Method B) at Drs. Aevitas Fertility Clinic.

Objectives: ***Primary objective***: To evaluate the effects of changing the embryo culture method on the blastulation outcomes. ***Secondary objective***: To evaluate the effects of changing the embryo culture method on the ART outcomes. ***Tertiary objective***: To determine whether the findings from this study support the continued use of Embryo Culture Method B at Drs. Aevitas Fertility Clinic for future ART cycles.

Methods and Materials: This was a retrospective study, utilizing the data obtained from the medical and laboratory records of Drs. Aevitas Fertility Clinic between January 2016 to December 2018. 479 cycles were included and separated into two sub-groups (Group A with 184 cycles and Group B with 295 cycles). All data were analysed and assessed for statistical significances ($p<0.05$) based on the difference in the means ± 95% confidence intervals.

Results: This study concluded that Group B attained statistically better blastulation outcomes than Group A, resulting in significantly higher blastocyst development rates [total blastocysts (53.96% vs. 40.70%), good-quality (11.97% vs. 4.45%), and fair-quality blastocysts (11.97% vs. 4.45%)], a significantly higher proportion of better-quality blastocysts [significantly more good-quality blastocysts (18.92% vs. 7.61%) and fewer poor-quality blastocysts (39.77% vs. 51.03%)], and a significantly higher day 5 embryo transfer rate (95.76% vs. 88.04%). Furthermore, Group B attained a significantly better blastocyst attribution profile, resulting in significantly more and better good-quality blastocysts obtained, utilized, and available for cryopreservation. This suggested a potential advantage of attaining better cumulative pregnancy rates than Group A. Group B further attained

slightly better ART outcomes, resulting in higher implantation rates (38.36% vs. 36.23%), higher clinical pregnancy rates (54.91% vs. 46.74%), lower miscarriage rates (9.15% vs. 10.33%), and higher live birth rates (47.12% vs. 41.85%). Although, no statistical significance was reported.

Conclusion: This study supports the continued use of Embryo Culture Method B at Drs. Aevitas Fertility Clinic in future ART cycles.

ABSTRAK

Agtergrond: Die embrio kultuursisteem speel 'n baie belangrike rol om die groei en ontwikkeling van die menslike embrio, in vitro, optimaal te maak. Studies wat hierdie kultuursisteme ondersoek is huidiglik skaars en ook soms teenstrydig, veral in die geval van watter die beste kultuurmedium (opeenvolgend vs aaneenlopend) en embrio kultuurmetode (individueel- vs saam kultuur) is. Elke laboratorium word dus aanbeveel om hul eie interne studies uit te voer om te bepaal watter kultuurkondisies vir hulle die beste kliniese uitkomste gee – gebaseer op hul unieke pasiëntpopulasie en laboratoriuminstelling.

Doelwit: Die studie het ondersoek ingestel na die effek wat die verandering van die embrio kultuurmetode A [waar embrios individueel kultuur is in "Sequential Series™ embryo culture medium (ORIGIO®)"] na embrio kultuurmetode B [waar embrios saam kultuur is in "SAGE™ 1-Step™ with Human Albumin Solution (ORIGIO®)"] teweeggebring het by die Drs Aevitas Fertiliteitskliniek.

Doel: ***Primêre Doel:*** Die evaluasie van die effek wat die verandering van die embrio kultuurmetode op blastosistvorming teweeggebring het***. Sekondêre Doel:*** Die evaluasie van die effek wat die verandering van die embrio kultuurmetode op geassisteerde reproduktiewe tegnieke (GRT) teweeggebring het. ***Tersiêre Doel:*** Bepaling of die bevindings van die studie die voortgesette gebruik van embrio kultuurmetode B vir tekomstige GRT siklusse by die Drs Aevitas Fertiliteitskliniek, ondersteun.

Metodes en Materiaal: Die huidige studie was retrospektief, die data wat gebruik is vir die ondersoek is verkry uit mediese- en laboratoriumdokumente van die Drs Aevitas Fertiliteitskliniek (Januarie 2016 tot Desember 2018). 479 siklusse is in die studie ingesluit. Die siklusse is onderverdeel in twee subgroepe (Groep A met 184 siklusse en Groep B met 295 siklusse). Alle data is analiseer en stastisties ontleed vir statistiese betekenisvolheid – gebaseer op die verskil in die gemiddeldes ± 95% vertrouensintervalle.

Resultate: Die studie se gevolgtrekking was dat blastosistvorming in Groep B statisties betekenisvol beter was as in Groep A. Blastosist ontwikkelingskoers [totale blastosiste (53.96% vs. 40.70%), goeie kwaliteit (11.97% vs. 4.45%) en billike kwaliteit (11.97% vs. 4.45%) was betekenisvol beter. Die proporsie van blastosiste met beter kwaliteit was ook groter. Betekenisvol meer goeie kwaliteit (18.92% vs. 7.61%) en betekenisvol minder swak kwaliteit blastosiste (39.77% vs. 51.03%) is gevind. Die dag 5 embrio terugplasingskoers (95.76% vs. 88.04%) was ook betekenisvol beter.

Daar is verder gevind dat Group B se toeskrywingsprofiel betekenisvol beter was. Betekenisvol meer goeie kwaliteit blastosiste is verkry, gebruik en was beskikbaar vir kriopreservering. Hierdie uitkoms dui daarop dat ‘n beter kumulatiewe swangerskapsyfer moontlik kan wees in Groep B. Alhoewel die GRT uitkomste nie statisties betekenisvol was nie, was alle GRT uitkomste gering beter in Groep B; beter implantasiesyfer (38.36% vs. 36.23%); beter kliniese swangerskapsyfer (54.91% vs. 46.74%); swakker miskraamsyfer (9.15% vs. 10.33%) en beter lewendige geboortesyfer (47.12% vs. 41.85%).

Gevolgtrekking: Die huidige studie se uitkoms ondersteun die voortgesette gebruik van embrio kultuurmetode B by die Drs Aevitas Fertiliteitskliniek vir toekomstige GRT siklusse.

DEDICATION

This thesis is dedicated to my son

Cole Bruce Mc Lachlan

without whom I would never have discovered my love for reproductive biology.

You remind me every day why this field is so important,

and the impact it can make on our patients' lives.

I will forever be grateful to have you in my life.

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TABLE OF CONTENTS

DECLARATION II
ABSTRACT III
ABSTRAK V
DEDICATION VII
AKNOWLEDGEMENTS VIII
LIST OF FIGURES 5
LIST OF TABLES 6
LIST OF ABBREVIATIONS 7
INTRODUCTION 9
CHAPTER 1: LITERATURE REVIEW 11
1.1 Infertility 13
1.2 Assisted reproductive technology 15
1.3 Factors affecting ART outcomes 17
1.3.1 Male and female diagnoses 17
1.3.2 Assisted reproductive techniques 18
1.3.3 Ova age 19
1.3.4 Number and maturity of ova 19
1.3.5 Methods and rates of fertilization 20
1.3.6 Day of embryo transfer 20
1.3.7 Number and quality of embryos transferred 21
1.4 Embryo culture systems 22
1.4.1 Culture media 22
1.4.2 Number of embryos per drop 29
1.5 Performance indicators 35
1.5.1 Blastulation outcomes 36
1.5.2 ART outcomes 38
1.6 Summary 40
AIM 41
RESEARCH QUESTIONS 42
OBJECTVES 43

HYPOTHESES 44

CHAPTER 2: MATERIALS AND METHODS 45

2.1 Study population 45

2.2 Study design 47

2.3 Data management and statistical analyses 48

2.4 Ethical aspects 50

2.4.1 Oocyte donation cycles 51

2.4.2 Waiver of consent 51

2.5 Methods 52

2.5.1 Controlled ovarian hyperstimulation 52

2.5.2 Oocyte retrieval and pick-up 52

2.5.3 Spermatozoa preparation 52

2.5.4 Oocyte insemination processes 52

2.5.5 Embryo culture 53

2.5.6 Blastulation evaluation 53

2.5.7 Embryo transfer 53

2.5.8 ART outcomes 54

2.6 Data analyses 54

2.6.1 Descriptive statistics 54

2.6.2 Blastulation outcomes 56

2.6.3 ART outcomes 58

CHAPTER 3: RESULTS 60

3.1 Patient population 60

3.2 Descriptive statistics 62

3.2.1 Male and female diagnoses 62

3.2.2 Assisted reproductive techniques utilized 64

3.2.3 Ova age 65

3.2.4 Number and maturity of ova 66

3.2.5 Methods and rates of fertilization 67

3.2.6 Number of embryos transferred 68

3.3 Blastulation outcomes 70

3.3.1 Blastocyst development rates 70
3.3.2 Proportion of various-quality blastocysts 71
3.3.3 Day 5 embryo transfer rates 72
3.3.4 Blastocyst attributions 73
3.4 ART outcomes 75
3.4.1 Implantation rates 75
3.4.2 Clinical pregnancy rates 76
3.4.3 Live birth rates 77
3.4.4 Miscarriage rates 78
CHAPTER 4: DISCUSSION 79
4.1 Study outcomes 79
4.4.1 Blastulation outcomes 80
4.1.2 ART outcomes 86
4.1.3 Evaluation of the embryo culture methods 90
4.2 Confounding factors 92
4.2.1 Male and female diagnoses 92
4.2.2 Assisted reproductive technique employed 93
4.2.3 Ova age 93
4.2.4 Number and maturity of oocytes obtained from aspiration 94
4.2.5 Fertilization rate 95
4.2.6 Day of embryo transfer 95
4.2.7 Number of embryos transferred 96
STRENGTHS AND LIMITATIONS OF STUDY 97
CONCLUSION OF STUDY 99
RECOMMENDATIONS FOR FUTURE STUDIES 100
REFERENCES 101
APPENDICES 109
A Controlled ovarian hyperstimulation 109
B Oocyst retrieval and pick-up 110
C Semen preparation 111
D Oocyte insemination 114
E Embryo culture and evaluation 120

F Blastocyst grading 122

G Embryo transfer 125

H Consent forms 127

I Disclosure of use of clinic's name, and institutional approval for use of patient and medical records 135

J Ethical approval 136

K Datasheet 137

L Statistical analyses 141

M Plagiarism report 161

LIST OF FIGURES

Figure 1.1	Factors impacting ART success	22
Figure 1.2	Common ingredients in sequential and continuous single-culture (monoculture; 1-step) media	27
Figure 2.1	Schematic of the study design	49
Figure 3.1	Flow chart indicating the data utilized for this study	61
Figure 3.2	Ova age	66
Figure 3.3	Number and maturity of ova	67
Figure 3.4	Fertilization rates	68
Figure 3.5	Number of embryos transferred	69
Figure 3.6	Blastocyst development rates	71
Figure 3.7	Proportion of various-quality blastocysts	72
Figure 3.8	Day 5 embryo transfer rates	73
Figure 3.9	Implantation rates	75
Figure 3.10	Clinical pregnancy rates	76
Figure 3.11	Live birth rates	77
Figure 3.12	Miscarriage rates	78

LIST OF TABLES

Table 1.1	Common male diagnoses determined from a routine semen analysis	14
Table 1.2	Common female diagnoses determined from infertility workup results	14
Table 2.1	Inclusion criteria	46
Table 3.1	Male diagnostic profiles	63
Table 3.2	Female diagnostic profiles	64
Table 3.3	Assisted reproductive techniques utilization profiles	65
Table 3.4	Blastocyst attributions	74

LIST OF ABBREVIATION

2PN	Two pronuclei
ACOG	American College of Obstetricians and Gynaecologists
AI	Artificial insemination
ART	Assisted reproductive technology
BDR	Blastocyst development rate
CLBR	Cumulative live birth rate
COC	Corona oocyte complex
CPR	Clinical pregnancy rate
EDTA	Ethylenediamine tetra-acetic acid
ESHRE	European Society of Human Reproduction and Embryology
ETR	Embryo transfer rate
FP	Forward progression
FSH	Follicle-stimulating hormone
GLPs	Good laboratory practices
GnRH	Gonadotropin-releasing hormone
GV	Germinal vesicle
HA	Hyaluronic acid
HCG	Human chorionic gonadotropin
HREC	Health Research Ethics Committee
ICM	Inner cell mass
ICMART	International Committee for Monitoring Assisted Reproductive Technology
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor
IMSI	Intracytoplasmic morphologically selected sperm injection
IR	Implantation rate
IU	International unit
IVF	In vitro fertilization
KPI	Key performance indicator
MI	Metaphase one
MII	Metaphase two

MR	Miscarriage rate
PAF	Platelet-activating factor
PB	Polar body
PGD	Pre-implantation genetic diagnosis
PGS	Pre-implantation genetic screening
PGT-A	Pre-genetic implantation testing for aneuploidy
PI	Performance indicator
PICSI	Physiological intracytoplasmic sperm injection
PN	Pronucleus
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SASREG	Southern African Society of Reproductive Medicine and Gynecological Endoscopy
SER	Smooth endoplasmic reticulum
SOP	Standard operating procedure
SWM	Sperm wash media
TE	Trophectoderm
WHO	World Health Organization

INTRODUCTION

Assisted reproductive technology has been greatly improved by optimizing the embryo culture system over the decades, resulting in the better growth and development of embryos *in vitro* (Thouas and Gardner, 2010, Sfontouris *et al*., 2016). However, studies reporting the optimal culture media (sequential media vs. continuous media) and method of embryo culture (individual culture versus group culture) are limited and contradictory (Paternot *et al*., 2010; Sfontouris *et al*., 2016; Morbeck *et al*., 2017). Therefore, although several economic, practical, and risk-associated advantages of co-culturing embryos in continuous media have been reported, there is still no clear consensus whether these potential optimizations will improve the patients' probability of ART success (Sfontouris *et al*., 2016; Werner *et al*., 2016; Deng *et al*., 2020). Thus, ART laboratories are recommended to perform their own internal studies to determine which of these culture conditions to obtain optimal clinical outcomes based upon their patient population and laboratory settings (Sfontouris *et al*., 2016; Morbeck *et al*., 2017; Deng *et al*., 2020).

Following these recommendations, this study was designed to determine whether Drs. Aevitas Fertility Clinic's decision to modify their embryo culturing system, from individually culturing embryos in sequential media (Embryo Culture Method A) to co-culturing embryos in continuous media (Embryo Culture Method B) in mid-2017 was beneficial.

This study, thus, retrospectively aims to evaluate the effects of changes in culture method over a three-year study period (January 2017-December 2018) and conclude if this study's findings support or refute the continued use of the Embryo Culture Method B, for future ART cycles, at Drs. Aevitas Fertility Clinic.

In doing so, **Chapter 1** will present an assembly of background information focused on infertility; ART utilized at Drs. Aevitas Fertility Clinic, the potential confounding factors considered, the embryo culture systems, and performance indicators routinely used to determine blastulation and ART outcomes. Finally, ending with the study's aim, research questions, objectives, and hypotheses.

Chapter 2 will present the study population, study design, data management, statistical analyses, ethical aspects, and methods and materials utilized in this study.

Chapter 3 will present the patient population, descriptive statistics, blastulation outcomes, and ART outcomes obtained, and statistical analysis thereof.

Chapter 4 will discuss the study's findings in the context of previously published literature.

The last chapter will discuss the study's strengths and limitations, conclusion, and recommendations for future studies.

CHAPTER 1

LITERATURE REVIEW

The following chapter includes an assembly of background information important for understanding and evaluating the aim of this study: to determine the effects of changing the embryo culture system (from individually culturing embryos in sequential media to co-culturing embryos in a continuous 1-step media) on the blastulation and assisted reproduction technology [ART] outcomes, at Drs. Aevitas Fertility Clinic, between January 2016 and December 2018.

The **first section** provides a *general overview of infertility*, discussing both its prevalence and causes.

The **second section** reports a *brief overview of ART*, discussing the common techniques employed at Drs. Aevitas Fertility Clinic in the treatment of their patients.

The **third section** describes the *factors affecting ART outcomes*, such as male and female diagnoses, the ART technique employed, the age, number, and maturity of ova obtained from aspiration, the methods and rates of fertilization, the day of embryo transfer, and the number and quality of embryos transferred. These factors are important to consider in preventing population bias.

The **fourth section** gives an *overview of the embryo culture system*. For the purpose of this study, two important factors will be discussed. Namely, the *type of culture media* utilized (sequential media versus continuous single-step culture media) and the *method of embryo culture* employed (individual culture versus group culture). These two factors directly affect the embryo culture environment by determining the components available to the embryos for growth. The type of media determines the components provided to the embryos by the embryologist, the method of embryo culture determines the components available to the embryos through the excretory and secretory factors produced by the embryos themselves.

The **fifth section** focuses on the *performance indicators* utilized to determine the effects of the change in the culture system on the *blastulation and ART outcomes*. More specifically, the *blastulation outcomes* measured in this study include the blastocyst development rate, the good blastocyst development rate, the proportion of good quality blastocysts, and the day five embryo transfer rate. On the other hand, the *ART outcomes* investigated include the implantation rate, the clinical pregnancy rate, the live birth rate, and the miscarriage rate.

The **sixth section** concludes this chapter and clarifies the study's *aim, research questions, objectives, and hypotheses*.

1.1 Infertility

Infertility, defined as the inability of a couple to achieve a pregnancy within one year of regular unprotected sexual intercourse, is estimated to affect approximately one in six couples during their reproductive years (Chui and Chamley, 2004; ESHRE, 2020). Globally, this equates to more than 150 million individuals at any given time (Inhorn and Patrizio, 2015). These individuals can be categorized into two groups: those presenting with primary infertility and those presenting with secondary infertility. Primary infertility refers to couples who have never achieved a pregnancy (Van der Merwe and Matsaseng, 2016). In comparison, secondary infertility refers to couples who have difficulty establishing a pregnancy after having conceived a child (Van der Merwe and Matsaseng, 2016).

The cause of the couple's infertility can usually be clinically assigned to either the male, female, or both partners (Van der Merwe and Matsaseng, 2016). Male infertility occurs in 20-30% of cases, female infertility occurs in 20-35% of cases, and both male and female infertility occurs in 25-40% of cases (ESHRE, 2020). In the remaining 10-20% of couples, the cause of infertility is unknown and reported as idiopathic (ESHRE, 2020). However, lifestyle-style related factors such as body weight, smoking, diet, exercise, physiological stress, caffeine or alcohol consumption, and exposure to environmental pollutants have been reported as potential contributing factors (Hofman *et al*., 2007; ESHRE, 2020).

Male infertility, more specifically, is either caused by pre-testicular, testicular, post-testicular, or immunological factors (Van der Merwe and Matsaseng, 2016). Although the cause of male infertility can help guide clinicians in their treatment strategy, male infertility is often easily overcome by employing an appropriate ART technique or utilizing a spermatozoa donor when necessary (Richardson *et al*., 2015). The chosen method depends on the male diagnosis, obtained from a standard semen analysis, and based on the patient's spermatozoa concentration, motility, and morphology (Table 1.1; WHO, 2010).

On the other hand, female infertility is determined from a woman's infertility workup results and is commonly caused by ovulatory dysfunctions, tubal disorders, a combination of factors, or idiopathic factors (Van der Merwe and Matsaseng, 2016). Although female infertility can be more challenging to overcome, depending on the female diagnosis, employing an appropriate ART technique, or utilizing an ova donor or surrogate, if necessary, can often result in achieving a live birth with sufficient ART treatments (Richardson *et al*., 2015; Table 1.2).

Table 1.1: Common male diagnoses determined from a routine semen analysis (adapted from WHO, 2010). Where two or more irregularities are identified the diagnoses are combined.

Diagnosis	Definition
Normozoospermia	The ejaculate contains a spermatozoa concentration, motility, and morphology above that of the lowest reference levels (spermatozoa concentration ≥15x10^6/ml; motility ≥32% and ≥40% for progressively motile and total motile spermatozoa respectively; morphology ≥4% normally formed spermatozoa).
Oligozoospermia	The ejaculate contains a spermatozoa concentration of <15x10^6/ml.
Asthenozoospermia	The ejaculate contains <32% progressively motile spermatozoa and/or <40% total motile spermatozoa.
Teratozoospermia	The ejaculate contains <4% normally formed spermatozoa.
Azoospermia	No spermatozoa are present in the ejaculate. This result should be confirmed from two separate ejaculations before the diagnosis is given.

Table 1.2: Common female diagnoses determined from infertility workup results.

Diagnosis	Definition
Advanced maternal age	The physiological state in which a woman presents with a significant reduction in her ovarian reserve (Child, 2013)
Recurrent miscarriage	The condition in which a woman presents with two or more consecutive losses of a fetus before 20 weeks of pregnancy (ACOG, 2020)
Anatomical factors	Irregularities in the woman's anatomy, which either impairs the progression of the oocyte, spermatozoa, and embryo's through the female reproductive tract, or structurally prevents the uterus from retaining a pregnancy (Hummelshoj *et al*., 2005).
Endocrine factors	A dysfunction of the woman's hypothalamic-pituitary-ovarian axis that adversely affects ovulation, such as anovulation or polycystic ovarian syndrome (Child, 2013).
Idiopathic infertility	The diagnosis assigned to a woman who is unable to conceive a child. This is despite having regular unprotected intercourse with a normozoospermic male and having normal fertility work-up results (Harrison and Taylor, 2006).

1.2 Assisted reproductive technology

Assisted reproductive technology [ART] refers to various specialized laboratory techniques and procedures implemented to treat infertility (Jones and Lopez, 2013). The primary objective of all ART treatments is to achieve a pregnancy in couples who were unable to receive corrective therapies (Jones and Lopez, 2013). Since the birth of the first *in vitro* fertilization [IVF] baby in 1978, ART has dramatically improved and resulted in the explosion of the industry (Jones and Lopez, 2013). Not only is it estimated that 2.4 million ART cycles are performed each year, giving rise to half a million babies, nowadays, more than half of couples who undergo treatment will successfully give birth to a child (Jones and Lopez, 2013; ESHRE 2020).

This success is largely attributed to the addition and optimization of ART techniques to overcome various infertility diagnoses (Kushnir *et al.*, 2017). These techniques include both *in vivo* techniques (in which fertilization occurs inside the body) and *in vitro* techniques (in which fertilization occurs outside of the body) (Child, 2013). The most common *in vivo* techniques include ovulation induction paired with timed intercourse or artificial insemination. Whereas *in vitro* techniques include in vitro fertilization [IVF] and various forms of intracytoplasmic sperm injection [ICSI] techniques such as traditional ICSI, physiological ICSI [PICSI], and intracytoplasmic morphologically selected sperm injection [IMSI] (Child, 2013).

When employing IVF, oocytes and spermatozoa are incubated together in fertilization media. In doing so, spermatozoa undergo the natural selection process to ensure only well-functioning spermatozoa fertilize the ova (Veeck and Zaninović, 2003; Harrison and Taylor, 2006). This method is recommended for couples where the male presents with normozoospermia or borderline oligozoospermia, which can be successfully overcome by concentrating the spermatozoa during semen preparation (WHO, 2010). Although some fertility clinics shy away from performing IVF only cycles due to an increased risk of fertilization failure, ESHRE (2020) has reported that IVF is still significantly employed and utilized globally in approximately 25% of all *in vitro* cases (Hojnik and Kovačič, 2019).

On the other hand, when employing ICSI, a single motile spermatozoon is selected, immobilized, and injected directly into the cytoplasm of an oocyte (Veeck and Zaninović, 2003). Although this method bypasses the natural selection of well-functioning spermatozoa, it enables embryologists to utilize spermatozoa with poorer parameters (Kruger, 2016). This advantage has increased its employment in fertility clinics worldwide, resulting in a utilization rate of about 75% of all *in vitro*

cases (ESHRE, 2020). However, it should be noted that this figure does not specify the subgroup of ICSI and thus includes both traditional ICSI and sub-methods such as PICSI and IMSI.

PICSI aims to select physiologically mature spermatozoa, which bind to hyaluronic acid [HA] drops fused to the bottom of the PICSI dish (Gatimel *et al*., 2016). This method is also reported to select for physiologically mature spermatozoa and those with better morphology and less DNA fragmentation, thus reducing possible chromosomal mutations (Beck-Fruchter *et al*., 2016). In comparison, IMSI aims to select the morphological best sperm by selecting the spermatozoa for ICSI injection using an interference contrast microscope at a high magnification of at least x6000 (Beck-Fruchter *et al*., 2016). In doing so, spermatozoa with nuclear vacuoles, which are reported to reduce nuclear quality, such as the degree of chromatin condensation, DNA integrity, and chromosomal content, can be deselected before injection (Gatimel *et al*., 2016). However, it should be noted that the benefit of employing PICSI and IMSI over traditional ICSI remains controversial (Beck-Fruchter *et al*., 2016; Gatimel *et al*., 2016).

1.3 Factors affecting ART outcomes

ART cycles are complex, with several confounding factors that can affect ART outcomes. When assessing a couple's prognosis or analysing the success of an ART method or procedure, factors such as (1) male and female diagnoses, (2) assisted reproductive techniques, (3) ova age, (4) number and maturity of ova, (5) methods and rates of fertilization, (6) day of embryo transfer, and (7) number and quality of embryos transferred, should be considered to prevent a population bias that can create incorrect associations (Skelly *et al*., 2012). Below is a summary of the potential confounding factors reported in previous relevant literature.

1.3.1 Male and female diagnoses

Male and female diagnoses identify physiological irregularities within the patient cohort (Gardener *et al*., 2008). Each diagnosis contains its own set of challenges and can affect the patients' fertility differently (Gardener *et al*., 2008). Some diagnoses can be directly overcome using ART. For instance, where *in vitro* fertilization techniques can be used to overcome the absence or blockage of the oviducts, and ICSI can be utilized to overcome severe oligozoospermia, asthenozoospermia, or teratozoospermia (Child, 2013; Kruger, 2016). Donors and surrogates can overcome infertility in couples where the patient's prognosis is extremely poor, or pregnancy is impossible (Child, 2013). The utilization of donor ova and spermatozoa has been successful in overcoming advanced maternal age in women with a poor ovarian response and azoospermia in men who either do not wish to undergo a testis biopsy or whose procedure is unsuccessful in obtaining spermatozoa (Practice Committee of the American Society for Reproductive Medicine & The Society for Male Reproduction and Urology, 2008; Child, 2013).

On the other hand, surrogates can be utilized to overcome the absence or abnormalities of the uterus that can prevent women from carrying a child (Child, 2013). However, in the case of other diagnoses, such as severe endometriosis, or in couples where the women are diagnosed with advanced maternal age but do not wish to use an ova donor, ART can be employed to increase the likelihood of achieving a pregnancy but does not remove the cause of infertility itself (Ozkan *et al*., 2008; Hojnik and Kovačič, 2019). Thus, the differences in a couple's diagnosis and chosen treatment plan should be carefully considered when comparing two populations.

1.3.2 Assisted reproductive techniques

The assisted reproductive technique [ART] employed can significantly affect cycle outcomes (Hojnik and Kovačič, 2019). This is especially important when comparing populations where the percentage of IVF and ICSI cycles performed are statistically different. It should, however, be remembered that this pertains to all ICSI techniques, as the utilization of specific ICSI techniques (such as traditional ICSI, physiological ICSI [PICSI], and intracytoplasmic morphologically selected sperm injection [IMSI]) to improve cycle outcomes is controversial with no definitive consensus in the results (Beck-Fruchter *et al.*, 2016; Gatimel *et al*., 2016).

When comparing IVF and ICSI cycles, it is well reported that ICSI cycles maintain a significantly higher fertilization rate (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017; Lee *et al*., 2017). This is attributed to the advantage of the technique in which a single spermatozoon is injected directly into the oocyte (Hojnik and Kovačič, 2019). In doing so, the effect of the spermatozoa's concentration, motility, and morphology is directly overcome and does not contribute to the selected spermatozoon's fertilization capability (Hojnik and Kovačič, 2019). On the other hand, successful IVF generally requires the male to present with normozoospermia, or borderline oligozoospermia, which can be overcome through the concentration of spermatozoa during the preparation process (Liu and Baker, 2000). Male factor infertility resulting in asthenozoospermia (poor motility) and teratozoospermia (poor morphology) can have a detrimental effect on the fertilization success of the oocytes (WHO, 2010). These factors increase the rates of total or near-total fertilization failure in IVF cycles, resulting in between 3-20% of all IVF cycles, compared to only 2-3% of ICSI cycles, and can significantly affect the overall fertilization rate of the ova within a population (Huang, 2015).

However, as the spermatozoa are manually selected in ICSI, the natural selection of the spermatozoa is forfeited. It is thought to contribute to a reported lower clinical pregnancy rate and live birth rate of those embryos which are fertilized with ICSI when compared to IVF cycles (Eftekhar *et al*., 2012). Therefore, the assisted reproductive technique employed, together with the male diagnoses, should be carefully considered when comparing two populations.

1.3.3 Ova age (female age at the time of oocyte retrieval)

Female fertility declines with age (Lawler *et al.* 2007; Liu and Case, 2011; Fleming *et al*., 2015). Although the speed at which fertility declines is patient-specific, in general, female fertility is expected to start declining slowly after 30 years old and more rapidly after 35 years old until menopause is reached (Fleming *et al*., 2015). This aging process significantly affects both the number and quality of oocytes obtained from an aspiration (La Marca *et al.*, 2017).

Not only does this reduce the number of ova available for fertilization as the woman's ovarian reserve is reduced, but an increase in the proportion of aneuploid oocytes. This, in turn, reduces the proportion of genetically normal oocytes that can be successfully fertilized and develop into euploid blastocysts (Lawler *et al.* 2007; Liu and Case, 2011; Fleming *et al*., 2015). Furthermore, research has strongly associated a reduction in the pregnancy rate and increase in the miscarriage rate, with an increase in female age from 37 years old onwards (Sauer *et al.*, 1990; Pirtea *et al*., 2020). This inverse relationship between female age and live birth rate emphasizes the need to consider the average ova age at the time of aspiration between populations studied.

1.3.4 Number and maturity of ova

Controlled ovarian hyperstimulation utilized in ART cycles aims to generate multiple mature oocytes obtained via ultrasound-guided aspiration (Law *et al*., 2019). The number and maturity of ova obtained to provide insight into the woman's ovarian reserve, response to stimulation, and prognosis of ART treatment (Committee of Gynaecologic Practice, 2015; Law *et al*., 2019). Several studies have indicated a strong positive association between the number of oocytes retrieved and the cumulative live birth rate [CLBR] (Melie *et al*.,2003; Sunkara *et al.*, 2011; Law *et al*., 2019). These studies reported that women with a normal ovarian response (4-25 ova per ART cycle) attained significantly higher CLBR than those presenting with either a poor ovarian response (1-3 ova per ART cycle) or excessive ovarian response (≥25 oocytes) (Melie *et al*.,2003; Sunkara *et al.*, 2011; Law *et al*., 2019).

These studies further suggested that a poor ovarian response is associated with a higher probability of obtaining immature ova, fertilization failure, cleavage failure, poor blastocyst quality, and poor ART outcomes (Melie *et al*.,2003; Sunkara *et al.*, 2011; Law *et al*., 2019). Contrarily, an excessive ovarian response is associated with inferior ova quality and reduced implantation rates (possibly resulting from diminished uterine receptivity) (Simón *et al*., 1995; Check *et al*., 1999).

Furthermore, in terms of oocyte maturity, patients with a high proportion of immature oocytes retain significantly lower fertilization rates, and a higher percentage of lower-quality blastocysts with an increased risk of complex mosaicisms and implantation failure (Lanzendorf *et al*.,1990; Reichman *et al*., 2010; Strassburger *et al*., 2010; Shin *et al*., 2013). As such, both the number and maturity of ova should be carefully considered when comparing two populations.

1.3.5 Methods and rates of fertilization

In an ART setting, the method of fertilization usually refers to either in vitro fertilization or intracytoplasmic sperm injection techniques (Beck-Fruchter *et al.*, 2016; Gatimel *et al*., 2016; ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017; Hojnik and Kovačič, 2019). Whereas the fertilization rate refers to the percentage of cumulus-oocyte-complexes (IVF) or metaphase II mature oocytes (ICSI) that are inseminated that go on to be normally fertilized (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

When comparing two populations, both the percentage of methods utilized, and the fertilization rates attained should be compared. This is important as the percentages of ART techniques utilized are expected to retain different fertilization rates (competency values of ≥60% for IVF and ≥65% for ICSI) (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Thereby affect both the blastulation and ART outcomes based on the number of zygotes obtained (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

Furthermore, differences in the fertilization rates (for each ART technique) gives insight into the good laboratory practices [GLPs] and technical competencies of embryologists attained over the study period (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). This is especially valuable for retrospective studies in which data for the two groups are collected sequentially (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

1.3.6 Day of embryo transfer

The day of embryo transfer, or more specifically, the duration for which the embryo is cultured, is another important confounding factor to consider. Although day 5 is generally the preferred time for embryo transfer, there are reported implications that longer *in vitro* incubation periods until the

blastocyst stage are associated with higher preterm delivery, larger gestational age babies, monozygotic twins, and altered sex ratio than the transfer of cleavage stage embryos (Maheshwari *et al.*, 2016). Furthermore, studies indicated that day 5 embryo transfers with the same number of blastocysts obtain a higher clinical pregnancy rate and live birth rate than Day 2 or 3 embryo transfers with cleavage-stage embryos (Glujovsky *et al.*, 2012). By extending the embryo culture protocol not only does it allow non-viable embryos, which have ceased development, to be identified and removed as an option for embryo transfer, but it enables the embryologists to gain more information regarding the prognosis of the remaining embryos based on their developmental rate and blastocyst grading (Gardner *et al.*, 2008; Glujovsky *et al.*, 2012). In doing so, the day of embryo transfer plays a significant role in ART outcomes.

1.3.7 Number and quality of embryos transferred

The number and quality of the embryos transferred are directly related to ART success (Pirtea *et al*., 2020). Thurin *et al.* (2004) reported that transferring two embryos significantly increased the clinical pregnancy rate compared to transferring a single embryo, from 39% to 43%. However, in doing so, it also increased the number of multiple births from 0.8% to 33%, thereby reducing the average gestational age and weights of infants born. However, when increasing the number of embryos to more than two, Ashrafi *et al.* (2015) indicated no significant improvement in the clinical pregnancy rates, but the reduction in the live birth rate, as the risk of gestational complications to both mother and fetuses was increased. The reduction in a live birth rate for multiple gestational pregnancies was also reported in a systematic review of the Cochrane Central Register of Controlled Trials in which elective single embryo transfers were associated with a higher live birth rate than that in which double embryo transfers were performed (McLernon *et al*., 2010).

In addition, the quality of the embryos transferred at the blastocyst stage has been reported to be a good predictor of pregnancy rate (Richardson *et al*., 2015). Good, fair, and poor-quality blastocysts report an incrementally significant reduction in their probability of implanting and achieving a clinical pregnancy and live birth (Richardson *et al*., 2015). Therefore, it is important to consider the number and quality of the embryos transferred for two populations.

1.4 Embryo culture systems

Assisted reproductive technology contains several impacting factors relating to the patient (such as ovarian stimulation, lifestyle factors, and genetics), the laboratory (such as the number and training level of the embryologists, number of incubators, and air quality), the culture system (such as the culture media used, and number of embryos cultured per drop), and the quality control and quality assurance measures put in place by an ART facility (Figure 1.1; Thouas and Gardner, 2010).

The success of ART we see today has resulted from the optimization of many of these individual factors (Dieamant *et al.*, 2017). Over the last few decades, researchers have reported dramatic improvements in the "culture system" or the elements that affect the embryos' growth and development (Ebner *et al.*, 2010; Thouas and Gardner, 2010). The two factors of the culture system, which are specifically important to this study, are: (1) the choice of culture media and (2) the number of embryos per drop.

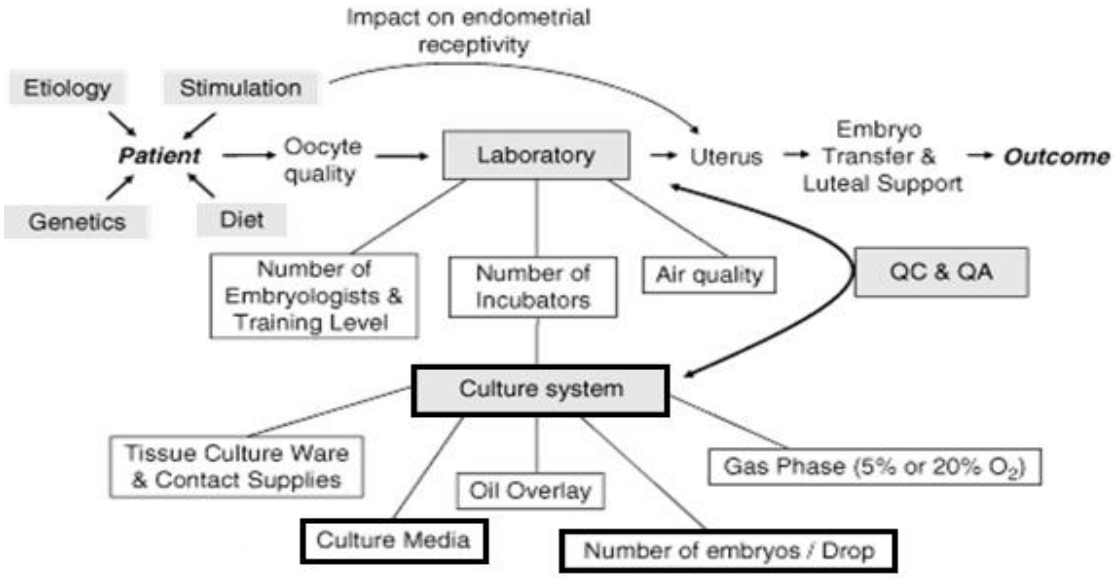

Figure 1.1: Factors impacting ART success (adapted from Thouas and Gardner, 2010).
The two culture system factors: the culture media and the number of embryos per drop, specifically important to this study, are emphasized in bolded blocks.

1.4.1 Culture media

The culture media refers to the choice of medium used to culture embryos from day 1 (post-fertilization) - day 5-7 (expected time of blastulation) (Sfontouris *et al.*, 2016). ART's success depends heavily on the quality of this embryo culture environment (Peak *et al.*, 2012; Morbeck *et al.*, 2017). In this review, the following three topics related to media will be discussed. Namely: (A)

the development of culture media over the decades, (B) the composition of sequential and continuous media, and (C) literature relating to the best embryo culture media to use in an ART laboratory.

A. The development of culture media over the decades

Culture media has dramatically changed over the last few decades (Sfontouris *et al*., 2016; Morbeck *et al*., 2017). When ART first began, embryos were cultured for 2 to 3 days (up to the 4- or 8-cell stage) in a somatic cell medium before being transferred back into the female's body (Biggers and Summers, 2008; Nagy *et al*., 2012). This media enabled embryologists to identify embryos that had been normally fertilized and were growing at the optimal speed with minimal signs of fragmentation or degradation (Maheshwari *et al*., 2016). Using this information, fertility clinics could transfer viable embryos back into the uterus. Although this culture method successfully resulted in the first IVF baby in 1978, it did not support extended embryo growth up to the blastocyst stage (Biggers and Summers, 2008). Therefore, as embryo cryopreservation had become a viable option for embryo utilization at a later date, and little was known about the implantation potential of the individual embryos, it was not easy to decide how many embryos should be used and how many should be discarded (Szeptycki and Bentov, 2016). To not waste potentially successful embryos, this resulted in a high number of embryos being routinely transferred back into the uterus, even though it increased the risk of multi-fetal pregnancies (Biggers and Summers, 2008).

However, in the 1990s, it became clear that clinicians required more information regarding the embryos' developmental potential (Biggers and Summer, 2008). Thus, there was a drive to increase the duration of embryo culture until pre-implantation (Biggers and Summers, 2008; Morbeck *et al*., 2017). Initial attempts utilized simple single-step media (such as Earle's balanced salt solution or T6 medium) (Sfontouris *et al*., 2016). However, these mediums resulted in disappointingly low implantation and pregnancy rates (Sfontouris *et al*., 2016).

Never-the-less, with continued research, the *back-to-nature approach* was developed and resulted in what we know today as *sequential media* (Dieamant *et al.*, 2017; Morbeck *et al*., 2017). Sequential media consists of two mediums, used in succession, to culture embryos post-fertilization (day 1) until blastulation (day 5-7) (Nagy *et al*., 2012). The first medium was developed to mimic the oviduct fluid, the natural environment for cleaving embryos (from day 1 to 3 of embryo culture). Whereas the second medium was developed to mimic the uterine fluids, the natural environment

for post-cleavage embryos (from day 4-7 of embryo culture) which are undergoing compaction or blastulation (Biggers and Summers, 2008; Dieamant *et al.*, 2017; Morbeck *et al*., 2017; Deng *et al*., 2020). In doing so, the embryos' changing metabolic and nutritional requirements were supported, while any build-up in toxin could be removed on day 3 (Biggers and Summers, 2008; Sfontouris *et al*., 2016; Cimadomo *et al.*, 2018). Furthermore, this improved the embryologist's ability to select embryos from the cohort with the best implantation potential, thereby reducing the need to transfer multiple embryos into the uterus. This reduced the risk of multi-fetal pregnancies while retaining clinical pregnancy rates of the ART facility (Biggers and Summers, 2008). In addition, embryos, which had undergone a day three blastomere biopsy, could be retained in culture until genetic results were processed. This enabled euploid embryos to be selected for a fresh embryo transfer on day 5, reducing time to pregnancy in many cases, especially women older than 38 years old (Biggers and Summers, 2008; Richardson *et al.*, 2015).

Shortly after the development of the first sequential media system, however, the possibility of developing a single medium which could support embryos, undisturbed using time-lapse technology, up until blastulation, became very appealing (Ciray *et al*., 2012; Sfontouris *et al*., 2016; Cimadomo *et al.*, 2018). This led to developing the first single embryo culture medium, known as the potassium simplex optimization medium, which could be used to continuously monitor murine embryos (Sfontouris *et al*., 2016). However, it was not long afterwards that it was proven that this medium could support human embryos (Sfontouris *et al*., 2016).

From here, continuous single-step culture media, used in many ART laboratories today, was developed to delicately balance the nutritional requirements of embryos undergoing cleavage, compaction, and blastulation (Ebner *et al*., 2010; Morbeck *et al*., 2017). While overcoming the need to remove toxic by-products of ammonium by substituting the previously used less-stable version of the amino acid glutamine with a more stable dipeptide form (either L-alanyl-L-glutamine or glycyl-L-glutamine) (Sfontouris *et al*., 2016).

With these changes, continuous mediums were reported to prevent the potential shock of transferring embryos from one media to the other and allow for the accumulation of embryo secreted autocrine and paracrine factors, an important consideration when using group culture (Section 1.4. ii) (Ebner *et al*., 2010; Sfontouris *et al*., 2016; Morbeck *et al*., 2017; Deng *et al*., 2020). In addition, clinical management teams reported an economic advantage resulting from the decrease in laboratory's labor requirements, a decrease in consumables (such as the media, dishes,

and pipettes), a decrease in staff labor and costs related to quality testing, and a decrease in unintentional human errors resulting from minimizing embryo handling time (Sfontouris *et al*., 2016; Deng *et al*., 2020). This was extremely advantageous for many laboratories, especially those with limited resources (Sfontouris *et al*., 2016). Furthermore, as mentioned previously, as the driving force behind this medium's development, compatibility with time-lapse technology was significantly beneficial for laboratories utilizing this technology (Sfontouris *et al*., 2016). However, it should be noted that that current evidence does not suggest a clear benefit of using time-lapse for embryo selection, even though it is reported to increase the overall costs of ART treatment (Sfontouris *et al*., 2016). With these advantages and the emergence of multiple studies that indicated that continuous media either improved or resulted in similar ART outcomes to that of the previously used sequential media, numerous laboratories quickly adopted this new media, regardless of the specific embryo culture incubators utilized (Biggers and Summers, 2008; Morbeck *et al*., 2017).

However, not all laboratories have adopted this media, with some laboratories reporting reduced ART results following the change to continuous media (Werner *et al*., 2016; Deng *et al*., 2020). Concerns mainly included the effects of not renewing nutrients and removing toxins on day 3, and the effects of evaporation within the embryo culture dish, as embryos are traditionally cultured in small volumes that are reported to undergo pH and osmolarity changes, even when an oil overlay and humidified incubators were being used (Biggers and Summers, 2008; Paternot *et al*., 2010; Nagy *et al*., 2012; Morbeck *et al*., 2017; Deng *et al*., 2020). As a result, there is still a debate about which media, sequential or continuous single-step media, is best to use in an ART setting (Section 1.4.1.C).

B. The composition of sequential and continuous single-culture media

There are currently two main types of culture media utilized in ART laboratories: sequential and continuous single-culture media (Figure 1.2). There are, however, multiple companies that have commercialized these media types resulting in various media compositions, with little information regarding the exact combination of constituents (Cimadomo *et al.*, 2018). Never-the-less, the media are reported to contain similar general components designed to mimic the *in vivo* environment (Nagy *et al*., 2012; Dieamant *et al*., 2017).

As previously discussed in Section 1.4.1.A, sequential media consists of two embryo development media (post-fertilization) (Nagy *et al*., 2012; Dieamant *et al.*, 2017; Morbeck *et al*., 2017). These

media are reported to retain crucial differences (Nagy *et al*., 2012). In Step 1, the first media, used to culture pre-compaction embryos (from day 1 to 3), contains non-essential amino acids, low-concentrations of (reduced) glucose, ethylenediamine tetra-acetic acid [EDTA], pyruvate, and lactate (Figure 1.2; Nagy *et al*., 2012). Whereas, in Step 2, the second media, used to culture post-compaction embryos (from Day 4 to 5), contains non-essential amino acids, essential amino acids, high concentrations (elevated) glucose, pyruvate, and lactate, while omitting EDTA (Figure 1.2; Nagy *et al*., 2012).

In Step 1, pyruvate and lactate are used by the cleavage staged embryos as a preferred energy substrate via the Krebs cycle, whereas trace glucose levels are used for intracellular signaling (Quinn, 2014). Before compaction (days 1-3), non-essential amino acids are used as chelators, pH regulators, precursors for protein formation, as an energy source, helping to maintain intracellular homeostasis, buffer the pH, act as an osmolyte, enhance early embryo development and cleavage, and function to reduce embryo stress. On the other hand, EDTA has been shown to overcome the 2-cell block (Biggers and Summers, 2008; Gardner *et al*., 2008; Nagy *et al*., 2012; Quinn, 2014).

In Step 2, glucose is elevated. It is used as the preferred energy source during glycolysis to provide enough energy for embryo compaction and blastocyst formation (Morbeck *et al*., 2017). Non-essential amino acids, together with the previously mentioned general functions, are also reported to support blastocoel formation, and are required for hatching. In contrast, essential amino acids also play a role in reducing embryo stress, stimulating the cleavage rate, and forming the inner cell mass (Gardner *et al.*, 2008). EDTA is, however, omitted from this media as it can have an adverse effect by suppressing glycolysis, which in turn results in fewer cells in the inner cell mass, and a significantly lower live birth rate (Nagy *et al*., 2012).

On the other hand, continuous single culture media consists of a single media, which has been designed to carefully balance the nutritional components required by the embryos for their entire development from post-fertilization until blastulation (Figure 1.2; Nagy *et al*., 2012). This media generally contains non-essential amino acids, essential amino acids, EDTA, glucose, pyruvate, and lactate. The role of these components is the same as above. However, interestingly, the presence of EDTA and glucose does not negatively affect the embryo's development due to the adjustment of these components. By significantly decreasing the concentration of EDTA usually found in sequential media, the 2-cell block can be overcome while reducing glycolysis suppression, allowing for the growth of the inner cell mass and an increase in live birth rates (Nagy *et al*., 2012).

Furthermore, the inclusion of sufficient amino acids concentrations into the media alleviates the adverse side effects of glucose before embryo compaction (Morbeck *et al*., 2017).

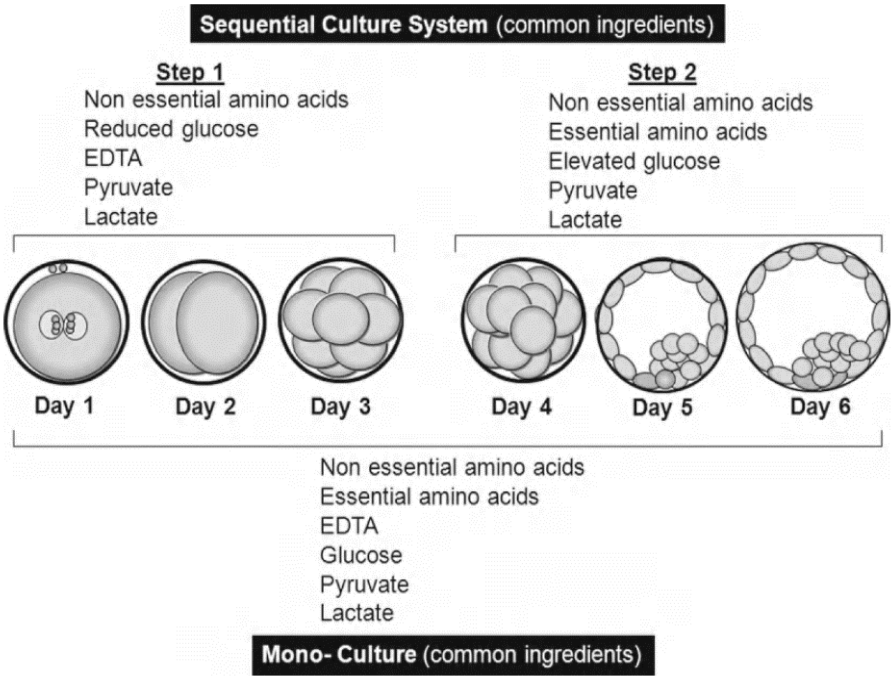

Figure 1.2: Common ingredients in sequential and continuous single-culture (monoculture; 1-step) media (obtained from Nagy *et al*., 2012).

C. Literature relating to the best embryo culture media to use in an ART laboratory

The evolution and optimization of embryo culture media have played a vital role in improving ART outcomes (Sfontouris *et al*., 2016). However, with numerous commercial culture media available and several studies indicating the adequate support of both sequential and continuous embryo culture media, there has been an interest in which culture system is superior and thus preferable to use within the ART laboratory (Paternot *et al*., 2010; Sfontouris *et al*., 2016). Although several studies comparing these media systems have been performed, very few were well-designed, and many utilized small sample sizes (Paternot *et al*., 2010; Sfontouris *et al*., 2016; Morbeck *et al*., 2017). This, unfortunately, led to several studies with limited outcomes as insufficient power calculation could not determine statistical significance (Paternot *et al*., 2010; Sfontouris *et al*., 2016; Morbeck *et al*., 2017).

However, a well-known meta-analysis study by Sfontouris *et al*. (2016) identified several randomized control studies comparing sequential media and continuous single-culture media in terms of blastulation and ART outcomes. Although the researchers reported an overall association

with an increase in the blastulation formation rate for the embryos cultured in a continuous single-step media there was no significant difference in the number of top-quality blastocysts and ART outcomes (such as the ongoing pregnancy rate, the clinical pregnancy rate, or the miscarriage rate). However, this may be a factor in reducing the number of studies and sample sizes considered. For instance, although ten studies (n=7455 oocytes/zygotes) reported results on the blastulation formation rate, only five of these studies went on to look at blastocyst quality, two studies (n=246 women) assessed the ongoing pregnancy rate and miscarriage rate, and just one study (n=100 women) reported the clinical pregnancy rate.

Similar results, reporting a higher blastulation rate for embryos cultured in continuous media, were indicated in several other studies.

Sepúlveda *et al*. (2009) reported that a single medium was as good as or better than a sequential media system for human embryo culture with a blastocyst development rate on day 5 of 42.9% vs. 31.1%. Furthermore, the implantation rate (72.5% vs. 57.9%) of those embryos transferred were significantly greater for embryos cultured in the continuous media. However, although there tended to be a higher clinical pregnancy rate (72.5% vs. 52.6%) and ongoing pregnancy rate (70.0% vs. 52.6%), these results were not statistically significant.

De la Calle *et al.* (2013) also reported a higher blastulation rate for embryos cultured in continuous media when compared to sequential media (51.1% vs. 40.7%). However, these researchers went on to also report no significant difference in the clinical pregnancy rates.

Deng *et al.* (2020) reported a significantly higher blastulation rate for embryos cultured in a SAGE 1-step medium as opposed to a SAGE advantage sequential medium (51.7% vs. 43.3%). However, these embryos also yielded a significantly higher aneuploidy rate (54.0% vs. 45.8%), resulting in an overall lower number of euploid embryos per cycle (2.6 vs. 3.3). However, for patients reaching euploid embryo transfer, there was no difference in the clinical pregnancy rates, miscarriage rates, or live birth rates between the two culture systems.

On the contrary, other studies reported contradictory results.

Werner *et al*. (2016) performed a randomized control trial, which indicated a higher blastulation rate for embryos cultured in sequential media as opposed to continuous media (55.2% vs. 46.9%).

However, these researchers went on to report that there was no significant difference in the aneuploidy rate and implantation rate of these embryos.

Several other studies reported no significant differences in both the blastulation rate and ART outcomes after culturing human embryos in either a sequential media or a continuous media, where applicable (Macklon, 2002; Ciray, 2012; Patrick, 2013; Sfontouris, 2015).

This range of results further emphasizes the recommendation for individual laboratories to perform their own internal studies to determine which media is best for both their general patient population and the limitations of their laboratory (Sfontouris *et al*., 2016; Morbeck *et al*., 2017; Deng *et al*., 2020).

1.4.2 Number of embryos per drop

The number of embryos per drop refers to the number of embryos incubated in each culture drop during the embryo developmental period (Reed *et al*., 2011). There are two main methods of culturing embryos: as ***individual culture***, where each embryo is cultured in a single drop of culture media, or as ***group culture***, where multiple embryos are cultured in each drop (Ebner *et al*., 2010).

Traditionally, ART employed individual culture (Ebner *et al*., 2010). This method has several advantages. First, embryo development can be tracked and monitored throughout the development process (Reed *et al*., 2011). This enables embryologists to track the embryo quality throughout development and select the embryo(s) with the highest implantation potential from the cohort. Second, it enables metabolic analyses to be performed on the embryo's surrounding media and used as part of the selection process (Rebollar-Lazaro and Matson, 2010). Third, it is extremely useful in separating oocytes or embryos with abnormalities (such as ova presenting with smooth endoplasmic reticulum [SER] disks or embryos presenting with only a single pronucleus) (Reed *et al*., 2011). Fourth, it enables embryologists to keep embryos in culture after performing procedures, such as embryo biopsy, which require unambiguous identification (Ebner *et al*., 2010; Reed *et al*., 2011). Lastly, it is well known that embryos can modify their surrounding environment by creating localized zones that enable optimal growth (Reed *et al*., 2011). Proponents of individual culture suggest that embryos, even within the same cohort, are unique. Thus, the embryos may grow better in culture unaffected by the secretome of surrounding embryos (Reed *et al*., 2011).

However, as ART progressed and researchers began optimizing embryo culture conditions, the number of embryos per drop was questioned, especially for poly-ovulatory species such as mice, rats, hamsters, and cats that naturally produce multiple ova and thus culture multiple embryos *in vivo* (Reed *et al*., 2011). Thus, the use of group culture to improve embryo culture *in vitro* in these species was investigated.

A study by Wiley *et al*. (1986) reported a significantly faster embryo development rate when twenty murine embryos were co-cultured together at a density of 0.5-0.6 µl/embryo.

Kelley and Gardner (2017) reported that single embryo culture was sub-optimal in mice resulting in a reduced number of inner cell mass cells at blastulation in embryos cultured individually (2 µl/embryo) either before or after compaction (mean of 112 and 110 cells respectively) when compared to those cultured at the same density but in groups of 10 embryos/20 µl culture drop (127 cells).

Moreover, Spindler and Wildt (2002) reported similar advantages in co-culturing results in felid embryos. However, they went on to investigate the effects of co-culturing embryos with different quality companion embryos. This study indicated that embryos cultured in groups of 10 embryos (2 µl/embryo) with equal or better-quality were reported to retain better developmental rates and consist of more cells per blastocyst (blastulation rate of 41.7% and 74.9 cells/embryo) than those cultured with poor-quality companion embryos (8.3% and 5.1 cells) or alone (8.3% and 8.4 cells/embryo).

The consensus was that group culture was beneficial for poly-ovulatory animals, and group culture became widely accepted for these species (Reed *et al*., 2011). In addition, several benefits of group culture were proposed. First, it was hypothesized that group-culture enabled the accumulation of embryo-trophic factors from good-quality embryos. These factors, commonly referred to as the secretome, are thought to consist of paracrine factors (targets nearby cells), autocrine factors (targets own cells), and endocrine factors (targets the circulatory system for ligand transportation) and can positively affect surrounding embryos within the beneficial zone (estimated at <160µM) (Rebollar-Lazaro and Matson, 2010; Reed *et al.*, 2011; Tao *et al*., 2013; Kelley and Gardner, 2017). To date, several of these factors, together with their role in improving ART success, have been identified. Insulin-like growth factor [IGF] I and II have been reported to help regulate cell growth, proliferation, and embryo survival (Ebner *et al*., 2010). Platelet-activating factor [PAF] has been

reported to reduce apoptosis and increase mitosis, ultimately impacting blastocyst cell number (Ebner *et al*., 2010; Agrogiannis *et al*., 2014). In addition, top-quality embryos have been shown to enhance the differentiation, decidualization, and secretion of chemokine and growth factors that promote the blastocyst's encapsulation and implantation by the endometrial epithelial cells (Sadeghi, 2017). These findings suggested that group culture was hypothesized to improve poorer-quality embryo development and implantation potential.

Second, group culture has an economic advantage. This culture method tends to be more affordable. Not only does it reduce the laboratory consumables by decreasing the number of micro drops required to be prepared for each cycle, but it can also reduce the number of culture dishes required for women from whom numerous ova are obtained. This is a worthwhile consideration for larger laboratories where the number of ova obtained from aspiration is routinely more than 8-10 and smaller laboratories with a limited budget. Furthermore, group culture can help reduce the overall dish preparation time by the embryologists. (Reed *et al*., 2011)

Contrarily, opponents of co-culturing embryos have reported several disadvantages to co-culturing human embryos. First, where good-quality embryos can positively affect the surrounding embryos, poor-quality embryos can negatively affect surrounding embryos. Therefore, co-culturing embryos with poorer-grade companions can be detrimental based on the embryo-toxic factors produced, which have been reported to inhibit the selective migratory response of decidualized endometrial stoma cells and subsequent active encapsulation of themselves (Reed *et al.*, 2011; Tao *et al*., 2013; Sadeghi, 2017). Second, group culture can potentially prevent individual embryos from optimally modifying their micro-environment as it is affected by the surrounding embryos (Gardner *et al*., 2008). Third, there is always a risk that co-culturing embryos may result in the depletion of essential nutrients, which could reduce the growth and development of surrounding embryos (Ebner *et al*., 2010; Reed *et al.*, 2011; Tao *et al*., 2013). Lastly, group culture is not recommended for patients who require dependable ova/embryo tracking. Such as those with abnormal oocytes/embryos or those who request embryo biopsy (Reed *at al*., 2011).

Nevertheless, with the emergent success of group-culture with poly-ovulatory species, studies on mono-ovulatory species (such as cows) and later humans were undertaken. Mono-ovulatory animal studies quickly showed success resulting in this culture method being generally accepted for use in animal species (Reed *at al*., 2011). For instance, Larson and Kubisch (1999) investigated the effects of co-culturing bovine embryos. The researchers reported superior blastulation outcomes from

embryos co-cultured at a density of 0.7 µl/embryo when compared to those individually cultured. More specifically, there was an increase in the blastocyst development rate (47.7% vs. 20.5%), an increase in the number of cells within the blastocyst (78.0 vs. 65.4), and the number of hatching blastocysts (26.9% vs. 12.8%).

However, results from human studies varied and did not show a clear consensus in embryo development or outcomes (Ebner *et al*., 2010; Reed *et al*., 2011). In early studies, researchers reported the effects of co-culturing cleavage stage embryos in groups on the embryo development/morphology and ART outcomes.

Moessner and Dodson (1995) reported a significant improvement in the cleavage rate (4.07 cells versus 3.11 cells) and embryo score (4.17 versus 3.90) of embryos co-cultured at 1-5 embryos/ml for 26 hours post-fertilization when compared to those which were individually cultured. There was, however, no significant difference in the embryo morphology.

Almagor *et al*. (1996) reported no significant difference in the mean number of blastomeres (both 4.3) for embryos cultured either individually or in groups of 3-5 normally fertilized embryos/700 µl of media. However, these researchers went on to determine ART outcomes. They reported an increase in the implantation (23% versus 11%) and pregnancy rates (43% versus 24%) for those embryos group cultured compared to those individually cultured and suggested that IVF outcomes were better with embryos undergoing communal growth.

Moreover, Spyropoulou *et al*. (1999) reported opposite ART results to Almagor *et al*. (1996). In their study, although embryos cultured either individually or in groups of between 3-5 embryos/20 µl for 1-2 days before day 2/3 fresh embryo transfer, had identical mean morphological scores, the embryos cultured individually, tended to have better ART outcomes, with an implantation rate (23% versus 21%) and clinical rate (20% versus 15%), when compared to those embryos co-cultured. However, the results were not statistically significant.

In later studies, researchers reported the effects of co-culturing embryos on the blastulation and ART outcomes.

Rebollar-Lazaro and Matson (2010) studied the effects of group culture of cleavage-stage embryos from day 1-3 before the individual culture of all embryos to the blastocyst stage. In this study, embryos were either cultured individually or in groups of 3 or more embryos in 15 µl of media. While

implantation rates and ongoing pregnancy rates were similar between groups, the blastocyst utilization rate of co-cultured embryos was significantly higher than those individually cultured (51.3% versus 46.5%) in women <35 years. Thus, the researchers suggested that it may be beneficial to increase the number of embryos cultured together for younger women.

Ebner *et al*. (2010), on the other hand, performed a prospective sibling study comparing individual and group culture from day 3 of culture until blastocyst stage (at a density of between 1-5 embryos/30 µl). On day 5, where possible, a single fresh embryo transfer was performed with the best quality blastocyst. For these patients, group culture was reported to be superior in terms of the percentage of clinical pregnancies (60.6% versus 30.0%) and the percentage of live births (60.5% versus 30.0%) compared to those embryos individually cultured. The researchers thus suggested that co-culturing pre-implantation embryos enhanced viability and should be utilized where possible.

On the other hand, Tao *et al.* (2013) studied the effect of co-culturing human embryos from day 3-5/6 at a density of 2-5 embryos per 50 µl culture drops, either randomly grouped or grouped according to quality (separated into either good quality embryos with ≥6 blastomeres, that were spherical and regular, with <15% fragmentation; or poor-quality embryos which did not meet these requirements). The study indicated that separating day 3 embryos into groups based on quality, positively affected blastocyst outcomes. More specifically, embryos grouped by quality promoted blastocyst development (61.2% vs. 44%) and blastocyst utilization rate (55.9% vs. 41.5%). Thus, increasing the number of embryos available for transfer and cryopreservation (4.5 vs. 2.7 embryos/patient). However, it should be noted that there were no significant differences in the implantation rates (36.7% vs. 41.5%) and clinical pregnancy rates (66% vs. 70%). The researchers suggested that this may have been related to a similar number of top-quality embryos transferred in both groups. Thus, the study concluded that although the pregnancy rate of the transferred embryos was equivalent, culturing embryos according to quality from day 3 may be beneficial in improving the overall quality of good-quality blastocysts cryopreserved for use in future cycles.

Thus, these human studies emphasize that there is no definitive consensus as to the effects of group culture on human embryo development and ART outcomes. However, from the literature currently available, it is generally accepted that although co-culture of human embryos may improve blastulation and ART outcomes, at worst, group culture will not negatively affect embryo development or ART outcomes (Reed *et al*., 2011; Tao *et al*., 2013). Therefore, it is recommended

that individual ART facilities perform their own studies to assess the potential advantages of co-culturing human embryos and determine whether this culture method should be incorporated into their standard operating procedures (Ebner *et al*. 2010; Reed *et al*., 2011).

1.5 Performance Indicators

Performance indicators [PIs], sometimes referred to as key performance indicators [KPIs], are a set of specific measures used to evaluate a method or procedure (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). All PIs should be objective, robust, reliable, easy to measure, and give both valuable and meaningful information regarding the efficacy or safety of the tested method or procedure (Pirtea *et al*., 2020).

In a fertility setting, PIs create valuable outcome markers to drive better results (within both the laboratory and/or clinical setting) (Pirtea *et al*., 2020). In doing so, not only do PIs allow for the excellence within the clinic by regularly monitoring and identifying shortfalls that need to be addressed, but it enables clinics to measure their internal levels of success with the success of other clinics both nationally and internationally (Pirtea *et al*., 2020). And thus, PIs do not only monitor and evaluate the laboratory's contribution to patient care, ensure the quality of the standard operating procedures are maintained, and perform external quality assurance and benchmarking, but it can also be a valuable tool by evaluating two methods or procedures and determining which method is preferable for the clinic and should be included within the clinic's SOPs (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

In 2017, specialists from the ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine met to establish the first comprehensive list, specifically for the ART laboratory, known as the Vienna Consensus. It consists of KPIs which evaluate oocyte collection, oocyte and sperm evaluations, fertility rates, embryo development, and freezing or treatment outcomes (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

More specifically, when evaluating a culture system, PIs related to embryo development (blastulation outcomes in the case of blastocyst-stage embryo transfer) and treatment outcomes (also known as ART outcomes) are important to consider. There are four reported blastulation outcomes: the (A) blastocyst development rate, (B) good blastocyst development rate, (C) proportion of good quality blastocysts, and (D) Day 5 embryo transfer rate; and four important ART outcomes: the (A) implantation rate, (B) clinical pregnancy rate, (C) live birth rate, and (D) miscarriage rate, when evaluating a new ART element. Below is a description of each of these PIs and information regarding how they can be useful in evaluating culture systems.

1.5.1 Blastulation Outcomes:

A. Blastocyst development rate

The blastocyst development rate, also referred to as the blastulation rate, is the proportion of normally fertilized oocytes (2PN zygotes) which go on to develop into blastocysts on either day 5 (116 ± 2 hours post-insemination), Day 6 (140 ± 2 hours post-insemination), or day 5 and 6 combined. This PI is considered an important blastulation outcome, as it provides important information regarding the effectiveness of the entire culture system to retain the viability of embryos throughout the culturing process and support embryo development from fertilization until blastulation. According to the Vienna consensus, laboratories should aspire to reach a competence and benchmark value of ≥40% and ≥60%, respectively. (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017)

However, laboratories should also remember that although this PI is considered an objective parameter, it relies on the use of well-defined blastocyst grading SOPs and the standardization of grading between embryologists, as the identification and inclusion of slow-growing blastocysts which have just begun cavitation are important to include. Furthermore, this PI also relies on consistent timing for all embryos between insemination and grading. Delayed grading may result in the addition of blastocysts, which would not have been included in the calculation. (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017)

B. Good blastocyst development rate

A good blastocyst development rate is defined as the proportion of normally fertilized oocytes (2PN zygotes) which go on to develop into a good-quality blastocyst on either day 5 (116 ± 2 hours post-insemination), Day 6 (140 ± 2 hours post-insemination), or day 5 and 6 combined (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Unlike the blastulation rate, however, this PI takes the blastocyst quality and stage into account. Blastocyst quality is based on the combination of the quality of three factors, the extent of blastocoele expansion, the appearance of the inner cell mass [ICM], and the appearance of the trophectoderm [TE] (Richardson *et al.*, 2015; ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). There are currently numerous methods, with varying degrees of intricacy used to classify blastocysts, namely good blastocysts, fair blastocysts, and poor blastocysts (Richardson *et al.*, 2015; ESHRE Special Interest Group of Embryology and Alpha Scientists in

Reproductive Medicine, 2017). Regardless of the grading system used, multiple studies have demonstrated a strong correlation between blastocyst quality and the blastocysts' prognostic potential to implant and achieve a clinical pregnancy and live birth (Richardson *et al.*, 2015). Richardson *et al.* (2015) developed clinically useful and simplified blastocyst grading methods, which results in statistically significant differences in the previously mentioned ART outcomes. This study indicates that good-quality blastocysts are fully expanded, have a prominent inner cell mass, and a cohesive trophectoderm, and are reported to have a 79.1 % implantation rate, 69.8% clinical pregnancy rate, and a 65.8% live birth rate.

According to the Vienna consensus, laboratories should aspire to reach a good blastocyst competence and benchmark value of ≥30% and ≥40%, respectively (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

However, laboratories should again remember that as with the blastocyst development rate that although this PI is considered an objective parameter, it also relies on consistent timing between insemination and grading, and consistency in grading to reduce both inter-observer and intra-observer variation. Furthermore, benchmark values can only be compared to clinics with similar grading systems (Richardson *et al*., 2015; ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

C. Proportion of good quality blastocysts

The proportion of good quality blastocysts is determined from the blastocyst development rate and the good blastocyst development rate (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Although a higher proportion of good quality blastocysts is not necessarily correlated to the success of obtaining a single live birth from an ART cycle, the proportion of good quality blastocysts may indicate the effectiveness of the culture system to more consistently support the development of blastocyst good quality blastocysts. The higher proportion of good quality blastocysts improves the implantation, clinical pregnancy, and live birth rate potential of each embryo, thereby improving the overall potential of the embryo cohort from the ART cycle (Richardson *et al.*, 2015). An increased number of good quality blastocysts, therefore, improves the chance of not only achieving a single live birth from the aspiration but further improves the potential of sibling live births in future years from the surplus cryopreserved embryos.

D. Day 5 embryo transfer rate

The day 5 embryo transfer rate is defined as the proportion of cycles with at least one 2PN zygote on day 1 with at least one blastocyst for transfer on day 5. This indicator is recommended for clinics that routinely perform day 5 transfers and reflect the entire culture system's effectiveness by determining the culture system's ability to sufficiently support the developing embryos to allow for the fresh embryo transfer to continue as planned. The Alpha survey indicated a large variation of competence and benchmark values ranging from 25 to 90% and 40 to 100%. It was suggested that the variation was related to differences in blastulation assessment and different grades of expansion. Therefore, the consensus is for each clinic to calculate its own reference values. (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017)

1.5.2 ART Outcomes:

A. Implantation rate

The implantation rate is defined as the percentage of transferred embryos that go on to implant and form a product of conception or fertilization, either a gestational sac, fetus, or fetal heart (Zegers-Hochschild *et al*., 2009). This indicator reflects the laboratory and culture system's overall performance, so an overall low implantation rate is a serious sign of a systematic problem. However, it should be remembered that this is not a stand-alone indicator. It also takes the uterine receptivity and the clinician's ability to transfer an embryo into account. Nevertheless, this indicator is important to consider when changing a specific element in the culture system. The Vienna consensus reports a competency and benchmark value for blastocyst-stage embryos' implantation rate as ≥35% and ≥60%, respectively. (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017)

B. Clinical pregnancy rate

The clinical pregnancy rate is defined as the percentage of pregnancies (gestational sac, fetus, or fetal heart) obtained from the total number of embryo transfers performed (Zegers-Hochschild *et al*., 2009). Therefore, it is similar to the implantation rate but looks at successful implantation per transfer as opposed to per embryo transferred.

This indicator can also reflect the laboratory and culture system's overall performance, although the clinician's ability to transfer an embryo and uterine receptivity are confounding factors (ESHRE

Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Despite this, this indicator is also important to consider when changing a specific element in the culture system.

C. Live birth rate

A live birth is defined as the complete expulsion or extraction from its mother of a product of fertilization, irrespective of the duration of the pregnancy, which, after such separation, breathes or shows any other evidence of life such as heartbeat, umbilical cord pulsation, or definite movement of voluntary muscles, irrespective of whether the umbilical cord has been cut or the placenta is attached (Zegers-Hochschild *et al*., 2009). Therefore, the live birth rate refers to the percentage of embryo transfers that go on to result in a live birth (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). This PI is often reported as an important PI of infertility studies. However, it should be remembered that this PI does not evaluate the health of the child after birth nor does it consider the confounding factors reflected as it does not only reflect the laboratory process as a whole, but also the clinical performance and gestational health.

D. Miscarriage rate

Lastly, a miscarriage is defined as the loss of an intrauterine pregnancy before 20 weeks of

gestation (Zegers-Hochschild *et al*.,2009). On the other hand, the miscarriage rate refers to the percentage of clinical pregnancies that result in a miscarriage (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). This may result in in a vanishing product of a clinical pregnancy in which there is a loss of gestational sac or embryo due to spontaneous disappearance. This PI again evaluates various confounding factors and reflects not only the laboratory but also the clinical performance. However, from a fertility clinic's perspective, it is useful to measure and evaluate the loss of clinical pregnancies, together with live births, to create a better understanding of the outcomes of the clinical pregnancies within the first 20 weeks of gestation.

1.6 Summary

At present, there is no clear consensus as to the benefits of co-culturing human embryos in 1-Step continuous media, as opposed to individually culturing embryos in sequential media (Ebner *et al*. 2010; Reed *et al*., 2011; Sfontouris *et al*., 2016; Morbeck *et al*., 2017; Deng *et al*., 2020). This large retrospective study thus aims to evaluate the change of the embryo culture system at Drs. Aevitas Fertility Clinic between January 2016 and December 2018 on the blastulation and ART outcomes. In doing so, the results obtained will either support or refute the continued use of co-culturing human embryos in 1-Step continuous media to improve the patients' probability of ART success.

AIM

The aim of this study is to investigate the effects of changing the embryo culture method at Drs. Aevitas Fertility Clinic, in 2017, between 2016 and 2018, from individually culturing embryos in Sequential Series™ embryo culture medium (ORIGIO®) (hereafter Embryo Culture Method A) to co-culturing embryos in SAGE™ 1-Step™ with Human Albumin Solution (ORIGIO®) (hereafter Embryo Culture Method B).

RESEARCH QUESTIONS

1. What was the effect of changing the embryo culture system from Embryo Culture Method A to Embryo Culture Method B on both the blastulation and ART outcomes at Drs. Aevitas Fertility Clinic?

2. Do the findings in (1) support the continued use of Embryo Culture Method B at Drs. Aevitas Fertility Clinic for future ART cycles?

OBJECTIVES

1. The **primary objective** of this study is to evaluate the effects of changing the embryo culture method on the blastulation outcomes at Drs. Aevitas Fertility Clinic. More specifically, to determine and compare the (i) blastocyst development rates (for both total blastocysts and various-quality blastocysts), (ii) proportion of various quality blastocysts, (iii) day 5 embryo transfer rate, and (iv) blastocyst attribution profiles (average number of blastocysts, obtained, transferred, and available for cryopreservation per ART cycle) for the two embryo culture methods used.

2. The **secondary objective** of this study is to evaluate the effects of changing the embryo culture method on the ART outcomes at Drs. Aevitas Fertility Clinic. More specifically, to determine and compare the (i) implantation rates, (ii) clinical pregnancy rates, (iii) live birth rates, and (iv) miscarriage rates for the two embryo culture methods used.

3. The **tertiary objective** is to compare both the blastulation and ART outcomes to determine whether the findings support the continued use of Embryo Culture Method B's at Drs. Aevitas Fertility Clinic, for future ART cycles.

HYPOTHESES

1. Embryo Culture Method B **improves blastulation outcomes** embryos by:
   (i) Increasing both the total and good-quality blastocyst development rates
   (ii) Increasing the proportion of good-quality blastocysts
   (iii) Increasing the day 5 embryo transfer rate
   (iv) Increasing the number of blastocysts obtained, transferred, and available for cryopreservation

2. If Embryo Culture Method B **improves the blastulation quality of the average embryo transferred**, Embryo Culture Method B is hypothesized to **improve ART outcomes** embryos by:
   (i) Increasing the implantation rate
   (ii) Increasing the clinical pregnancy rate
   (iii) Increasing the live birth rate, and
   (iv) Decreasing the miscarriage rate

3. The findings of this study **support the continued use of Embryo Culture Method B** at Drs. Aevitas Fertility Clinic for future ART cycles.

CHAPTER 2

METHODS AND MATERIALS

2.1 Study population

The study population selected for this study included all individuals who met the inclusion criteria (Table 2.1). This was regardless of their infertility diagnoses or decision to use a donor or surrogate.

In summary, these individuals were all patients at Drs. Aevitas Fertility Clinic, Pinelands, South Africa, between January 2016 and December 2018. Their treatments utilized either in vitro fertilization [IVF], intracytoplasmic sperm injection [ICSI], physiological ICSI [PICSI], or intracytoplasmic morphologically selected sperm injection [IMSI] and resulted in a day 5 embryo transfer. Only fresh assisted reproduction technology [ART] cycles were considered. All frozen ova cycles, frozen embryo cycles, and freeze all cycles were excluded to prevent the confounding factors related to cryopreservation. Furthermore, all cycles utilizing pre-implantation genetic screening [PGS] or pre-implantation genetic diagnosis [PGD] were excluded. This decision was made to prevent the confounding factors related to artificially breaching the zona pellucida during the biopsy procedure.

All patients included in this study, also utilized ova (autologous or donor) obtained from women who ≤38 years old and presented with good ovarian stimulation (≥4 ova obtained from ova retrieval). Furthermore, all cycles resulted in at least one normally fertilized zygote. Lastly, all patients included in this study were available for follow-up to obtain ART outcomes.

Table 2.1: Inclusion criteria

The criteria utilized to include patients in this study.

Criteria	Inclusion
Treated at Drs. Aevitas Fertility Clinic (2016-2018)	Yes
In vitro fertilization technique employed	Yes
Ova age (years)	≤38
Number of ova retrieved	≥4
Number of normally fertilized zygotes	≥1
Day 5 embryo transfer performed	Yes
Frozen ova or frozen embryos utilized	No
PGS or PGD employed	No
Follow-up ART outcomes obtained	Yes

2.2 Study design

This study was retrospectively conducted on the data obtained from medical and laboratory records between 2016 and 2018 at Drs. Aevitas Fertility Clinic, Pinelands, South Africa (as depicted in Figure 2.1). Following the selection of the study population (Section 2.1), a total of 479 cycles were included. These cycles were further separated into two groups, based on the embryo culture method utilized. Group A consisted of 184 cycles that included cycles in which embryos were individually cultured in the Sequential Series™ embryo culture medium (ORIGIO®) (hereafter, Embryo Culture Method A) (performed from January 2016 to mid-2017). Whereas Group B consisted of 295 cycles that included cycles in which embryos were co-cultured in SAGE™ 1-Step™ with Human Albumin Solution (ORIGIO®) (hereafter, Embryo Culture Method B) (performed from mid-2017 to December 2018). The effects of culturing embryos using the two embryo culture methods could be determined and compared by assessing both blastulation outcomes (primary objective) and ART outcomes (secondary objective).

The blastocyst outcomes assessed, included the (i) blastocyst development rates (for both total blastocysts and various-quality blastocysts), (ii) proportion of various quality blastocysts, (iii) day 5 embryo transfer rate, and (iv) blastocyst attribution (average number of blastocysts, obtained, transferred, and available for cryopreservation per ART cycle) for the two embryo culture methods used. Whereas, the ART outcomes assessed, included (i) the implantation rates, (ii) the clinical pregnancy rates, (iii) the live birth rates, and (iv) the miscarriage rates.

After which, the study findings could be assessed to determine whether this study supports the continued use of Embryo Culture Method B at Drs. Aevitas Fertility Clinic, for future ART cycles (tertiary objective).

2.3 Data management and statistical analyses

Data for this study was obtained from the standard, routine data files from Drs. Aevitas Fertility Clinic. Relevant anonymized data was transferred to a Microsoft Excel spreadsheet specifically designed for statistical analyses (Appendix K). A statistician from the Division of Epidemiology and Biostatistics, Stellenbosch University, used appropriate statistical methods to analyse the data. Descriptive statistics, blastulation outcomes, and ART outcomes were calculated from the data represented by Group A and Group B (Section 2.2). Two variants of statistical analyses depending on the data were utilized in this study. Where Group A and B profiles were assessed (male and female diagnostic profiles and ART utilization profiles), Pearson Chi-square tests ($P<0.05$) were utilized to identify statical differences. However, where differences in the sample mean were assessed (ova age, number and maturity of ova, fertilization rate, number of embryos transferred, blastocyst development rates, the proportion of various quality blastocysts, blastocyst attribution, implantation rates, clinical pregnancy rates, live birth rates, and miscarriage rates), two-sample t-tests assuming equal variance ($P<0.05$) were performed. In each case, the mean ± 95% confidence interval range was reported. Statistically significant means were either depicted using asterisks (in tables) or by placing the significant p-values directly above the relevant bars on the graphs (in figures). For more information regarding the statistical analyses, please refer to Appendix L.

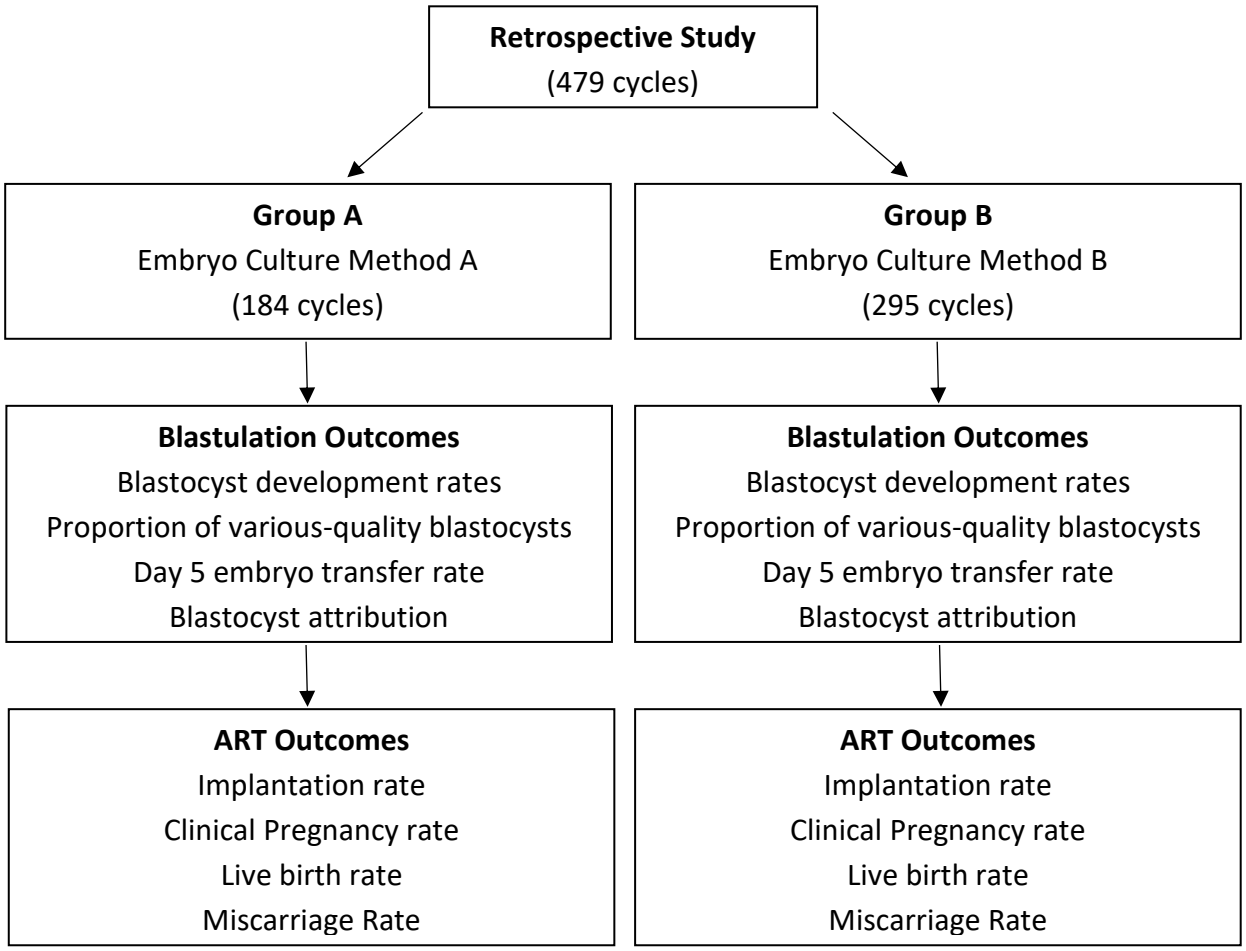

Figure 2.1: **Schematic of the study design**

479 ART cycles, performed between January 2016 and December 2018, were included in this study, and separated into two groups. Group A consisted of 184 cycles and utilized Embryo Culture Method A. Group B consisted of 295 cycles and utilized embryo Culture Method B. For both groups, the blastulation outcomes (blastocyst development rates, proportion of various-quality blastocysts, day 5 embryo transfer rate and blastocyst attribution) and ART outcomes (implantation rate, clinical pregnancy rate, live birth rate, and miscarriage rate) were determined and compared.

2.4 Ethical aspects

As, this is a retrospective study, patients were not subjected to any additional risks, injury, or pain through its completion. All data was obtained with the clinic's consent and further indicated the institutional approval and agreement to disclose the clinic's name in this study (Appendix I). Furthermore, to retain patient confidentiality, a senior embryologist from Drs. of Aevitas Fertility Clinic transferred the relevant data from the clinic's database to a password-protected Microsoft Excel spreadsheet. Here, the embryologist allocated a unique arbitrary number to each cycle (not linked to any file number or identifier) to allow for future internal tracking of patients only by investigators at Drs. Aevitas Fertility Clinic, if necessary. The senior embryologist also filtered the data to remove all unique identifiers such as name, identification numbers, contact information, treatment date, and date of birth). Following these changes, the embryologist handed over the secured unidentifiable data spreadsheet and password, for use in this study.

Once obtained, only a single saved version of this file was preserved on a secure device. Back-ups were periodically given to the senior embryologists for retrieval in case of an emergency. This version was adapted to only contain the required information, which included the patients' sex (male or female), age, patient diagnosis (related to infertility as this is an exclusion criterion), method of *in vitro* fertilization (IVF, ICSI, PICSI, or IMSI), number of follicles obtained from aspiration, media used for embryo culture (sequential media or continuous culture medium), method of culturing embryos (single or group culture), number of embryos fertilized, number of embryos that undergo blastulation, quality of blastocysts, number of embryos transferred, presence or absence of a biochemical pregnancy, number of clinical pregnancies, absence or presence of a miscarriage, number of live births, the gestational age of children born and infant birth weights. In doing so, the only demographic information utilized in this study was the patients' genders, patients' infertility diagnoses and female patients', ova donors' and surrogates' ages. Patient demographics such as their name, identification number, clinic number, date of birth, nationality, race, postal code, income, education, contact information, marital status, diseases (not related to infertility diagnosis) were not included. Furthermore, to minimize the risk of a data breach, only the final, non-traceable version of this data was handed over for

analyses by the statistician. In addition, all data presented in this thesis was aggregated to prevent the presentation of individual results.

2.4.1 Oocyte donor cycles

In cycles where donor ova were required, oocytes donors, from a Southern African Society of Reproductive Medicine and Gynecological Endoscopy [SASREG] accredited oocyte donor agency, donated oocytes to the recipient couple. Donor oocytes were fertilized with the spermatozoa of the male partner of the recipient couple, and the resultant good-quality blastocysts were either transferred or cryopreserved. There is a legal contract between the donor and the receiving couple. This contract stipulates that the donor has no legal claim to the embryos. Therefore, the oocyte donors gave consent to donate their oocytes to a recipient, and the recipient gave consent to transfer and/or vitrify the resultant blastocysts after fertilization (Appendix H).

2.4.2 Waiver of consent

This study evaluated retrospective data obtained from the medical and laboratory records from Drs. Aevitas Fertility Clinic on completed clinical procedures. For this reason, the Health Research Ethics Committee [HREC] has waived informed consent as indicated as part of the ethical approval for this study (HREC Reference number: S19/09/188] (Appendix J).

2.5 Methods

The methods employed by clinicians and reproductive scientists were followed according to the standard operating procedure [SOP] of Drs. Aevitas Fertility Clinic, as described below.

2.5.1 Controlled ovarian hyperstimulation

Female patients were stimulated according to the SOP of Drs. Aevitas Fertility Clinic. Ovarian stimulation occurred with the administration of medication, which stimulated the ovaries to produce multiple oocytes through follicular development, as described in Appendix A.

2.5.2 Oocyte retrieval and pick-up

Standard oocyte retrieval and pick-up procedures were performed according to the SOP of Drs. Aevitas Fertility Clinic (Appendix B). Briefly, follicular fluid was aspirated utilizing sonar guided ultrasound, and examined for the presence of corona-oocyte-complexes [COC] both macroscopically and microscopically. The oocytes within the COCs were superficially graded to infer maturity (immature, germinal vesicles [GV] or metaphase I [MI] oocytes; mature, metaphase II [MII]). Post grading, the COCs were collected, washed in Quinn's Advantage™ Medium with HEPES (SAGE™), and transferred to a Fert™ (ORIGIO®) medium.

2.5.3 Spermatozoa preparation

Semen was processed using standard, routine protocols as described in the SOP of Drs. Aevitas Fertility Clinic (Appendix C). Both the swim-up and discontinuous gradient centrifugation preparation methods were utilized, depending on the semen characteristics.

2.5.4 Oocyte insemination

Oocytes were inseminated using standard protocols for in vitro fertilization [IVF], intracytoplasmic sperm injection [ICSI], physiological intracytoplasmic sperm injection [PICSI], or intracytoplasmic morphologically selected sperm injection [IMSI], as described in the SOP of Drs. Aevitas Fertility Clinic (Appendix D).

2.5.5 Embryo culture and evaluation

Embryos were cultured in PLANER CO_2 benchtop incubators (ORIGIO®) using the standard SOPs of Drs. Aevitas Fertility Clinic (Appendix E). However, as the SOP for embryo culture was modified in mid-2017, two embryo culture methods were employed during this study. These changes made up the basis of this study and were carefully considered when data was collected, grouped, and analysed. Briefly, cycles occurring prior to the modification of the embryo culture SOP, where placed into Group A, and utilized the Embryo Culture Method A, in which embryos were individually cultured in the Sequential Series™ embryo culture medium (ORIGIO®). Whereas cycles occurring after the modification of the embryo culture SOP, where placed in Group B, and utilized Embryo Culture Method B, in which embryos were co-cultured in SAGE™ 1-Step™ with Human Albumin Solution (ORIGIO®). Furthermore, during the first 3 days of embryo culture (prior to blastocyst evaluation) fertilization checks and embryo evaluations were performed as described in Appendix E.

2.5.6 Blastocyst evaluation

Blastocyst evaluation was performed, based on a modification of the Schoolcraft *et al*. (1999) method, as described in Appendix F. Briefly all blastocysts were morphologically evaluated based on their cell expansion, appearance of their inner cell mass, and appearance of their trophectoderm. For this study, blastocyst grading was obtained on day 5 of cell culture at approximately 116 ± 2 hours post-insemination. These blastocysts were further categorized into one of three blastocyst quality categories, namely good blastocysts, fair blastocysts, and poor blastocysts. This categorisation method was modified from Richardson *et al*. (2015) and further described in Appendix F.

2.5.7 Embryo transfer

A standard embryo transfer was performed by transferring embryo(s) into the uterus utilizing sonar guidance, as described in the SOP of Drs. Aevitas Fertility Clinic (Appendix G). In general, one to three embryos were selected for embryo transfer depending on the woman's age, number of previous failed ART cycles, and quality of the blastocysts available for transfer. Embryos were

selected based on their grading hierarchy, where embryo with the best grade is selected first, and the embryo with the worst grade is selected last. This hierarchy is determined by grading the blastocysts based on the appearance of their expansion, trophectoderm, and inner cell mass as described in Appendix F.

2.5.8 ART outcomes

ART outcomes were obtained through follow-up sessions with the patients. During these sessions, relevant medical information was acquired, such as ultrasound images of gestational sacs and fetuses or medical reports stating the expulsion of fetuses or the number of children born. This information was further utilized to report the implantation rate and absence or presence of a clinical pregnancy, live birth, or miscarriage, for each patient (as specified in Section 2.6.3).

2.6 Data analyses

Data was analysed in three sections: (1) descriptive statistics, (2) blastulation outcomes, and (3) ART outcomes.

2.6.1 Descriptive statistics

Descriptive statistics were utilized to determine whether the two populations (Group A and B) were balanced and comparable (Mann, 1995; Skelly *et al*., 2012). In this study, the following six confounding factors were considered: (A) male and female diagnoses, (B) ART method utilized, (C) ova age, (D) number and maturity of ova, (E) fertilization rate, and (F) number of embryos transferred.

A. Male and female diagnoses

The male and female diagnostic profiles for Group A and B were compared to determine whether the percentage of each diagnosis was similarly represented in each group. In doing so, the following equation was used:

$$Diagnosis\ (\%) = \frac{Number\ of\ cycles\ with\ a\ specified\ diagnosis}{Number\ of\ ART\ cycles} \times 100$$

B. ART method utilized

The ART utilization profile for Group A and B was compared to determine whether the percentage of each ART technique (IVF, ICSI, or a combination thereof) was similarly represented in each group. Thus, the following equation was used:

$$ART\ method\ (\%) = \frac{Number\ of\ cycles\ utilizing\ a\ specific\ ART\ method}{Number\ of\ ART\ cycles} \times 100$$

C. Ova age

The mean ova age for Group A and B was determined using the following equation:

$$Ova\ age\ = \frac{Sum\ of\ the\ women's\ ages\ undergoing\ oocyte\ aspiration}{Number\ of\ ART\ cycles}$$

D. Number and maturity of ova

The mean number and maturity of ova for Group A and B was determined using the following equation:

$$Number\ of\ ova = \frac{Sum\ of\ the\ total\ or\ specified\ maturity\ ova\ obtained\ from\ aspirations}{Number\ of\ ART\ cycles}$$

E. Fertilization rate

For this study, the fertilization rate is defined as the proportion of mature ova that were normally fertilized (presented with 2 pronuclei) on day 1 of embryo culture following insemination. The mean fertilization rate for Group A and B was calculated using two equations. First, the fertilization rate for each ART was determined. After which, the mean fertilization rates for the two groups could be determined and compared.

$$Fertilization\ rate\ (\%)\ = \frac{Number\ of\ 2PN\ zygotes\ on\ day\ 1}{Number\ of\ MII\ ova\ at\ time\ of\ insemination} \times 100$$

$$Mean\ fertilization\ rate\ (\%)\ = \frac{Sum\ of\ fertilization\ rates\ (\%)}{Number\ of\ ART\ cycles}$$

F. Number of embryos transferred

The mean number of embryos transferred for Group A and B was determined using the following equation:

$$Number\ of\ embryos\ transferred\ = \frac{Sum\ of\ the\ embryos\ transferred}{Total\ number\ of\ ART\ cycles} \times 100$$

2.6.2 Blastulation outcomes

Blastocyst outcomes were utilized to determine the efficiency of the culture system to routinely produce blastocysts (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). The blastulation outcomes selected for this study included: (A) blastocyst development rates, (B) proportion of specific-quality blastocysts, (C) day 5 embryo transfer rates, (D) the attribution of blastocysts. These outcomes were determined and compared for patients in Group A and Group B.

A. Blastocyst development rates

The blastocyst development rate [BDR], also referred to as the blastulation rate, is defined as the proportion of normally fertilized zygotes on day 1, that underwent blastulation on day 5 (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). The following equation was used to determine the BDRs for Group A and Group B:

$$BDR = \frac{Number\ of\ blastocysts\ on\ day\ 5}{Number\ of\ 2PN\ zygotes\ on\ day\ 1} \times 100$$

In addition, the specific-quality BDRs, were further be determined by calculating the BDR for each specific-quality of blastocyst. This study categorizes blastocysts into three categories: good, fair, and poor-quality blastocysts (Appendix F). The following equation was used to determine the specific-quality BDR for Group A and Group B:

$$< Quality > \ BDR = \frac{Number\ of\ < quality > blastocysts\ on\ day\ 5}{Number\ of\ 2PN\ zygotes\ day\ 1} \times 100$$

B. Proportion of specific-quality blastocysts

The proportion of blastocysts categorized into specific categories is expressed as the proportion of all day 5 blastocysts with a specified quality (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). The following equation was used to determine the proportion of specified quality blastocysts for Group A and Group B:

$$Proportion\ of\ < qulaity > blastocysts =$$

$$\frac{Number\ of\ \ < quality > \ blastocysts\ on\ day\ 5}{Total\ number\ of\ blastocysts\ on\ day\ 5} \times 100$$

C. Day 5 embryo transfer rate

The day 5 embryo transfer rate [ETR] is defined as the proportion of cycles with at least one normally fertilized zygote (containing 2PN) on day 1 (17 ± 1 hour post insemination) that had at least one blastocyst for transfer on day 5 (116 ± 2 hours post-insemination) (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). The following equation was used to determine the day 5 ETRs for Group A and Group B:

$$Day\ 5\ ETR = \frac{Number\ of\ cycles\ with\ \geq\ 1\ blatocyst\ on\ day\ 5}{Number\ ocycles\ with\ \geq\ 1\ 2PN\ zygote\ on\ day\ 1} \times 100$$

D. Attribution of blastocysts

The attribution of blastocysts refers to the average number of blastocysts: obtained (from aspirations), utilized (during day 5 fresh embryo transfers), and remained in culture following embryo transfers (and thus were available for cryopreservation), per ART cycle. The following equation was used to determine both the mean number of blastocysts (and the mean number of specific-quality blastocysts) within each category, for Group A and Group B:

$$Mean\ number\ of\ blastocysts = \frac{Sum\ of\ blastocysts\ within\ each\ category}{Total\ number\ of\ ART\ cycles}$$

2.6.3 ART outcomes

ART outcomes were utilized to determine the long-term outcomes of those embryos transferred (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). The ART outcomes selected for this study included: (A) implantation rates, (B) clinical pregnancy rates, (C) live birth rates and (D) miscarriage rates. These rates were determined and compared for patients in Group A and Group B.

A. Implantation rates

Implantation refers to the attachment of a blastocyst to the uterine lining. Successful implantation can be observed through the ultrasonographic visualization of a gestational sac (Zegers-Hochschild *et al*., 2009). Implantation rate [IR], therefore, refers to the rate at which transferred embryos successfully implant. The following equation was used to determine the implantation rate for each patient.

$$IR = \frac{Number\ of\ gestational\ sacs}{Number\ of\ embryos\ transferred} \times 100$$

B. Clinical pregnancy rates

A clinical pregnancy is determined by ultrasonographic visualization of one or more gestational sacs, or any other definitive sign of a clinical pregnancy, such as a positive fetal heartbeat or ectopic pregnancy (Zegers-Hochschild *et al*., 2009). A clinical pregnancy rate [CPR] can, therefore, be expressed as the number of clinical pregnancies per one hundred embryo transfer cycles performed (Zegers-Hochschild *et al*., 2009). The following equation was used to determine the CPR for Group A and Group B:

$$CPR = \frac{Number\ of\ clinical\ pregnancies}{Number\ of\ embryo\ transfers} \times 100$$

It should, however, be noted that a pregnancy resulting in multiple gestational sacs or multiple fetuses should only be counted as a single clinical pregnancy.

C. Live birth rates

A live birth is defined as the complete expulsion or extraction from its mother of a product of fertilization, irrespective of the duration of the pregnancy, which, after such separation, breathes or shows any other evidence of life such as heartbeat, umbilical cord pulsation, or definite movement of voluntary muscles, irrespective of whether the umbilical cord has been cut or the placenta is attached (Zegers-Hochschild *et al*., 2009). A live birth rate is thus the number of deliveries that resulted in at least one live baby expressed per one hundred embryo transfer cycles (Zegers-Hochschild *et al*., 2009). The following equation was used to determine the LBR for Group A and Group B:

$$LBR = \frac{Number\ of\ live\ birth\ deliveries}{Number\ of\ embryo\ transfers} \times 100$$

It should, however, be noted that the live birth rate resulting in the birth of multiple live babies should only be counted as a single live birth.

D. Miscarriage rates

A miscarriage is defined as the loss of an intrauterine pregnancy before 20 weeks of gestation (Zegers-Hochschild *et al*.,2009). A miscarriage rate [MR] can be defined as the number of clinical pregnancies resulting in a miscarriage expressed per one hundred clinical pregnancies. The following equation was used to determine the MR for Group A and Group B:

$$MR = \frac{Number\ of\ miscarraiges}{Number\ of\ clinical\ pregnancies} \times 100$$

It should be noted that a miscarriage resulting in the loss of multiple fetuses should only be counted as a single miscarriage. Furthermore, in cases where a multiple pregnancy, results in the disappearance of one or more fetuses through reabsorption of fetal tissue but continues to on to a life birth of one or more remaining fetuses, this pregnancy is reported as one clinical pregnancy, one miscarriage and one live birth delivery.

CHAPTER 3
RESULTS

The results of this study are presented in Figures 3.1-3.12 and Tables 3.1-3.4.

3.1. Patient population

The data utilized in this study was obtained from the patient medical records of Drs. Aevitas Fertility Clinic between 01 January 2016 and 31 December 2018. From the 2 489 assisted reproductive technology [ART] cycles performed; 2 010 cycles were removed based on the exclusion criteria (Section 2.2). This resulted in the inclusion of 479 cycles, which were further separated into two groups depending on the embryo culture method employed. Group A consisted of 184 cycles and utilized embryo Culture Method A (where embryos were individually cultured in sequential media). Group B consisted of 295 cycles and utilized embryo Culture Method B (where embryos were co-cultured in 1-Step continuous media (Figure 3.1).

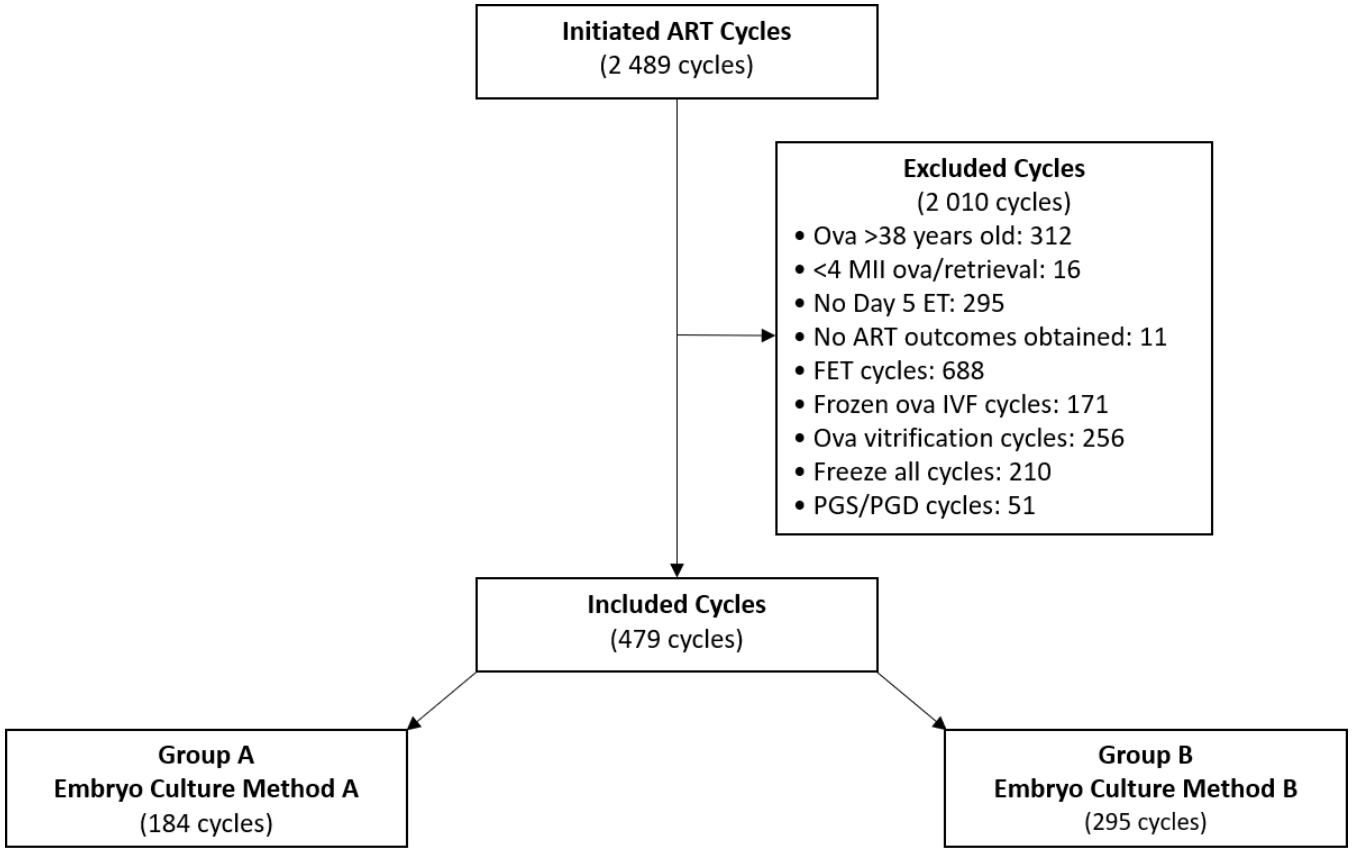

Figure 3.1: Flow chart indicating the data utilized for this study

2489 ART cycles were initiated at Drs. Aevitas Fertility Clinic between January 2016 and December 2018. 2010 of these cycles were excluded from this study. 479 cycles were included and separated into two groups depending on the embryo culture method utilized. Group A consisted of 184 cycles and utilized embryo Culture Method A (where embryos were individually cultured in sequential media). Group B consisted of 295 cycles and utilized embryo Culture Method B (where embryos were co-cultured in 1-step continuous media).

ART, Assisted reproductive technology; D5, Day five; FET, frozen embryo transfer; IVF, in vitro fertilization; MII, Metaphase two; PGD, preimplantation genetic diagnosis; PGS, Preimplantation genetic screening.

3.2 Descriptive statistics

Descriptive statistics were performed on the study population to ensure that Group A and Group B were evenly balanced and comparable. Based on the current relevant literature (summarized in Section 1.3), the following potential confounding factors were considered: male and female diagnoses, assisted reproductive techniques utilized, ova age (age of the female undergoing the oocyte aspiration), number and maturity of ova, fertilization rate, and the number of embryos transferred.

3.2.1 Male and female diagnoses

The male and female diagnostic profiles for Group A and B are presented in Tables 3.1 and 3.2, respectively.

In the case of the male diagnoses, 65.22% of men in Group A presented with normal fertility parameters, whereas 34.78% presented with male-factor infertility. More specifically: 62.55% of men were diagnosed with normozoospermia, 9.78% with oligozoospermia, 1.63% with asthenozoospermia, 7.07% with teratozoospermia, 5.97% with a combination of oligozoospermia/ asthenozoospermia/teratozoospermia, and 10.33% with azoospermia (but from whom testicular spermatozoa were successfully obtained and utilized for ova insemination).

Similarly, 66.10% of the men in Group B presented with normal fertility parameters, whereas 33.90% presented with male-factor infertility. Here, 66.10% of males presented with normozoospermia, 11.19% with oligozoospermia, 1.69% with asthenozoospermia, 7.12% with teratozoospermia, 6.10% with a combination of oligozoospermia/asthenozoospermia/ teratozoospermia, and 7.80% with azoospermia (but again from whom testicular spermatozoa were successfully obtained and utilized for ova insemination).

In the case of the female diagnoses, 40.23% of women in Group A presented with normal fertility parameters, whereas 59.76% presented with female-factor infertility. More specifically, for couples presenting with normal fertility parameters: 19.57% presented with male-factor infertility only, 19.57% with idiopathic infertility, and 1.09% without a male partner (female couples/individuals only). On the other hand, for those presenting with female-factor infertility: 32.07% were diagnosed with ovarian insufficiency (utilized donor ova), 0.54% with ovarian insufficiency (utilized autologous

ova), 9.24% with endometriosis, 8.15% with an anatomical factor, 5.98% with an endocrine factor, 2.72% with recurrent miscarriage, and 1.09% required a surrogate.

Similarly, 31.19% of women in Group B presented with normal fertility parameters, whereas 68.81% presented with female-factor infertility. More specifically, for couples presenting with normal fertility parameters: 15.93% presented with male-factor infertility only, 11.53% with idiopathic infertility, and 3.73% without a male partner (female couples/individuals only). On the other hand, for those presenting with female-factor infertility: 40.00% were diagnosed with ovarian insufficiency (utilized donor ova), 0.68% with ovarian insufficiency (utilized autologous ova), 8.14% with endometriosis, 9.15% with an anatomical factor, 7.80% with an endocrine factor, 2.03% with recurrent miscarriage, and 1.02% required a surrogate.

The differences between the diagnostic profiles for Group A and Group B were assessed using Pearson Chi-squared tests ($P<0.05$). No significant differences were obtained for both the male ($p=0.3233$) and female ($p=0.2244$) diagnostic profiles.

Table 3.1: **Male diagnostic profiles**

Male diagnoses are reported as a percentage of the total male patient cohort for each group. Group A (n=184) consisted of ART cycles in which embryos were individually cultured in sequential media. Group B (n=295) consisted of ART cycles in which embryos were co-cultured in 1-step continuous media. A Pearson Chi-square test was performed on the diagnostic profiles for Group A and Group B ($P<0.05$). No statistical differences were obtained between the two profiles ($p=0.3233$).

Diagnosis	Group A	Group B
Normal male-fertility parameters	**65.22%**	**66.10%**
Normozoospermia	65.22%	66.10%
Male-factor infertility	**34.78%**	**33.90%**
Oligozoospermia	9.78%	11.19%
Asthenozoospermia	1.63%	1.69%
Teratozoospermia	7.07%	7.12%
Oligo-asthenozoospermia	0.54%	3.73%
Oligo-teratozoospermia	3.26%	1.69%
Astheno-teratozoospermia	0.54%	0.34%
Oligo-astheno-teratozoospermia	1.63%	0.34%
Azoospermia (testicular spermatozoa utilized)	10.33%	7.80%

Table 3.2: **Female diagnostic profiles**

Female diagnoses are reported as a percentage of the total female patient cohort for each group. Group A (n=184) consisted of ART cycles in which embryos were individually cultured in sequential media. Group B (n=295) consisted of ART cycles in which embryos were co-cultured in 1-step continuous media. A Pearson Chi-square test was performed on the diagnostic profiles for Group A and Group B ($P<0.05$). No statistical differences were obtained between the two profiles ($p=0.2244$).

Diagnosis	Group A	Group B
Normal female-fertility parameters	**40.23%**	**31.19%**
Male-factor infertility	19.57%	15.93%
Idiopathic infertility	19.57%	11.53%
No male partner (sperm donor required)	1.09%	3.73%
Female-factor infertility	**59.76%**	**68.81%**
Ovarian insufficiency (donor ova utilized)	32.07%	40.00%
Ovarian insufficiency (autologous ova utilized)	0.54%	0.68%
Endometriosis	9.24%	8.14%
Anatomical factor	8.15%	9.15%
Endocrine factor	5.98%	7.80%
Recurrent miscarriage	2.72%	2.03%
Surrogate utilized	1.09%	1.02%

3.2.2 Assisted reproductive techniques utilized

The assisted reproductive technique utilization profiles for Group A and B are presented in Table 3.3.

In this study, ICSI (ejaculated spermatozoa) refers to all ICSI techniques utilizing spermatozoa obtained via ejaculation. These techniques included traditional ICSI, physiological ICSI [PICSI], and intracytoplasmic morphologically selected sperm injection [IMSI]. In comparison, ICSI (testicular spermatozoa) refers specifically to traditional ICSI utilizing testicular spermatozoa, obtained following a testis biopsy.

In Group A, 1.63% of all cycles employed IVF only, compared to 77.72% of cycles that employed ICSI techniques (of which 66.85% of cycles utilized ejaculated spermatozoa and 10.87% utilized testicular spermatozoa). The final 20.65% of cycles utilized a combination of both IVF and ICSI techniques.

Similarly, in Group B, 1.02% of all cycles employed IVF only, compared to 82.71% of cycles that employed ICSI techniques (of which 74.91% of cycles utilized ejaculated spermatozoa and 7.80%

utilized testicular spermatozoa). The final 16.27% of cycles utilized a combination of both IVF and ICSI techniques.

The differences between the ART utilization profiles for Group A and B were assessed using Pearson Chi-squared tests (P<0.05). No significant differences in ART utilization profiles were obtained between the two groups (p=0.2873).

Table 3.3: Assisted reproductive techniques utilization profiles

The ART utilization profiles in Group A and B are reported as percentages of the total number of cycles performed for each group. Group A (n=184) consisted of cycles in which embryos were individually cultured in sequential media. Group B (n=295) consisted of cycles in which embryos were co-cultured in 1-step continuous media. A Pearson Chi-square test was performed on the ART profiles for Group A and Group B (P<0.05). No statistical differences were obtained between the two profiles (p=0.2873).

Assisted reproductive techniques	**Group A**	**Group B**
***In vitro* fertilization techniques**	**1.63%**	**1.02%**
IVF	1.63%	1.02%
Intracytoplasmic sperm injection techniques	**77.72%**	**82.71%**
ICSI (ejaculated spermatozoa)	66.85%	74.91%
ICSI (testicular spermatozoa)	10.87%	7.80%
Mixed techniques	**20.65%**	**16.27%**
IVF and ICSI techniques (ejaculated spermatozoa)	20.65%	16.27%

3.2.3 Ova age

The average ova age (age of the female undergoing the oocyte aspiration) for Group A and B are presented in Figure 3.2.

The mean ova age per ART cycle for Group A and Group B was 30.60 ± 0.75 years and 30.27 ± 0.64 years, respectively. A two-sample t-test assuming equal variance (P<0.05) was performed and indicated that there was no significant difference in the mean ova age between the two groups (p=0.5261).

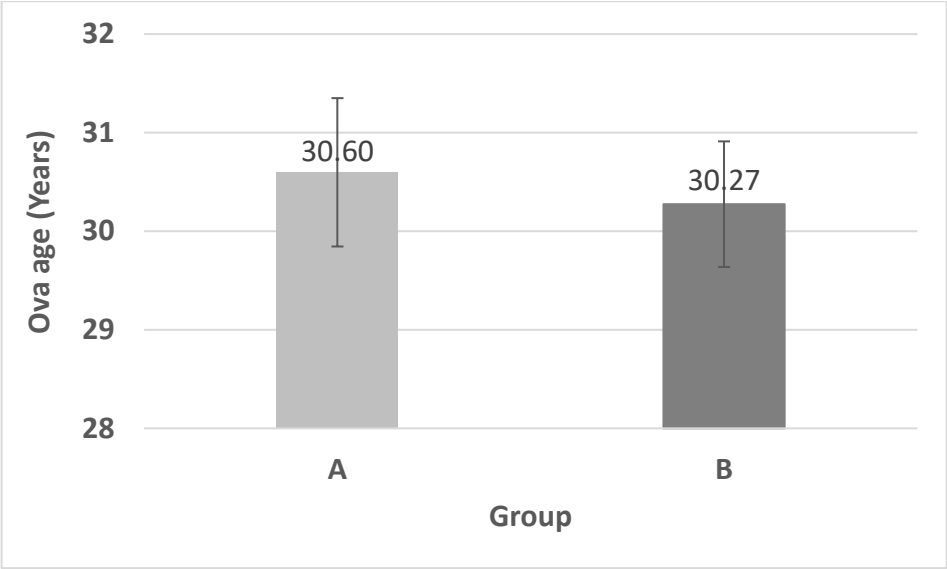

Figure 3.2: Ova age
Ova age was determined as the mean age of the women undergoing the oocyte aspiration for Group A (n=184) in which embryos were individually cultured in sequential media and Group B (n=295) in which embryos were co-cultured in 1-step continuous media. A two-sample t-test assuming equal variance ($P<0.05$) was performed and inferred no statistical significance between the means ($p=0.5261$). The error bars represent the 95% confidence interval of the sample mean.

3.2.4 Number and maturity of ova

The total number of ova (regardless of maturity) and number of ova (separated by maturity), for Group A and B, are presented in Figure 3.3(i) and Figure 3.3(ii), respectively.

In this study, the total number of ova (regardless of maturity) refers to the average number of ova obtained from the women undergoing oocyte aspiration. In contrast, the number of ova (separated by maturity) refers to the average number of both mature and immature ova obtained per oocyte retrieval prior to insemination. All metaphase II [MII] ova were classified as mature ova. Whereas all metaphase I [MI] oocytes or oocytes containing germinal vesicles [GV], were classified as immature ova.

The mean number of ova in Group A was 12.68 ± 1.06 (consisting of 10.82 ± 0.92 mature ova and 1.86 ± 0.36 immature ova). Whereas the mean number of ova in Group B was 13.08 ± 0.70 ova (consisting of 11.53 ± 0.64 MII and 1.55 ± 0.20 immature ova). Two-sample t-tests assuming equal variance ($P<0.05$) indicated that there was no significant difference in the mean number of total ova ($p=0.5232$), mature ova ($p=0.2002$), nor immature ova ($p=0.1063$) between the two groups.

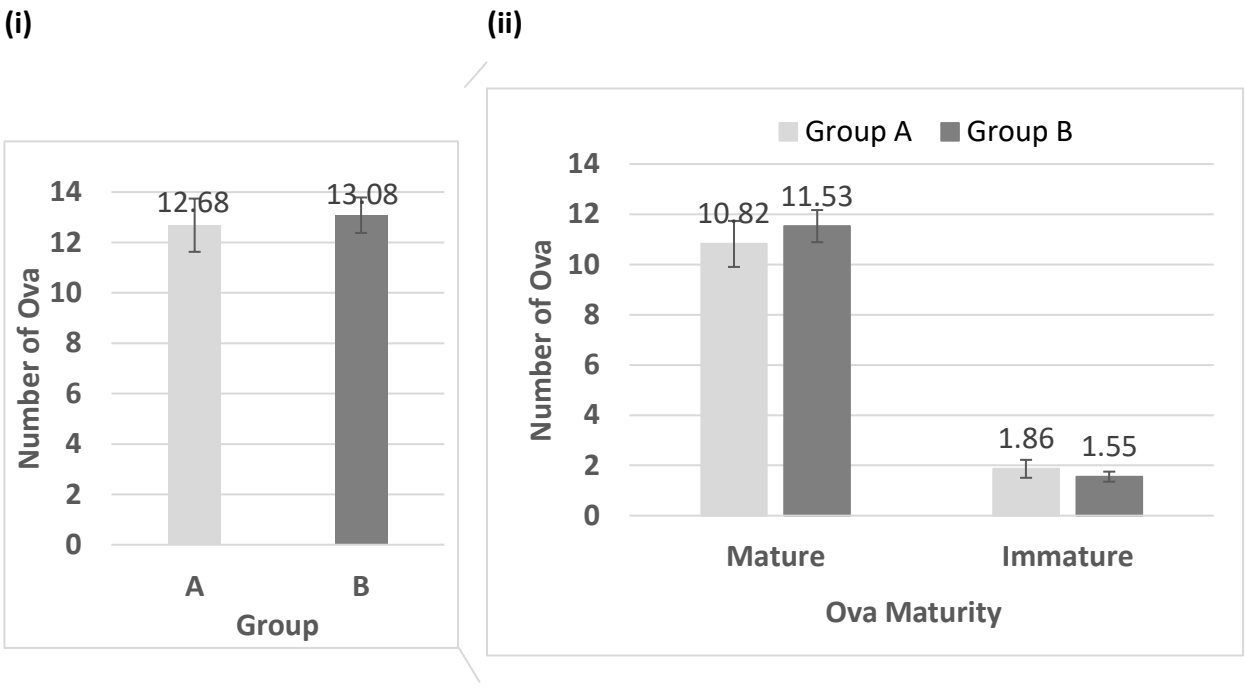

Figure 3.3: Number and maturity of ova

(i) The total number of ova (regardless of maturity) per ART cycle obtained for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media). **(ii)** The number of ova (separated by maturity) per ART cycle for Group A (n=184) and Group B (n=295). Mature ova included all metaphase II ova, and immature ova included all metaphase I ova and ova containing germinal vesicles at the time of insemination. Two-sample t-tests assuming equal variance were performed ($P<0.05$) and inferred no statistical differences between the means of the total ova ($p=0.5232$), mature ova ($p=0.2002$), nor immature ova ($p=0.1063$). The error bars represent the 95% confidence interval of the sample mean.

3.2.5 Methods and rates of fertilization

The average fertilization rate for Group A and B are presented in Figure 3.4.

In this study, the fertilization rate was defined as the percentage of mature ova observed to be normally fertilized 16-18 hours following IVF or ICSI insemination. This definition was adapted from the Vienna Consensus to incorporate the fertilization rate from both IVF and ICSI cycles into a single value for Group A and Group B (ESHRE Special Interest Group of Embryology and Alpha Scientists Reproductive Medicine, 2017). The decision was based upon no significant difference observed between the ART utilization profiles for Group A and B, the vast majority of cycles for both groups utilizing ICSI techniques only (77.72% versus 82.71%), very few cycles for both groups utilizing IVF only (1.64% for Group A and 1.02% for Group B), and insufficient information being obtained during data capturing regarding the specific number of ova inseminated via IVF or ICSI that went on to present with two pro-nuclei in the mixed cycles (20.65% versus 16.27%) (Section 3.2.2).

The mean fertilization rates in Group A and B were 82.25 ± 4.94% and 80.83 ± 1.98%, respectively. A two-sample t-test with equal variance (P<0.05) indicated that there was no significant difference in the average fertilization rate between the two groups (p=0.5483).

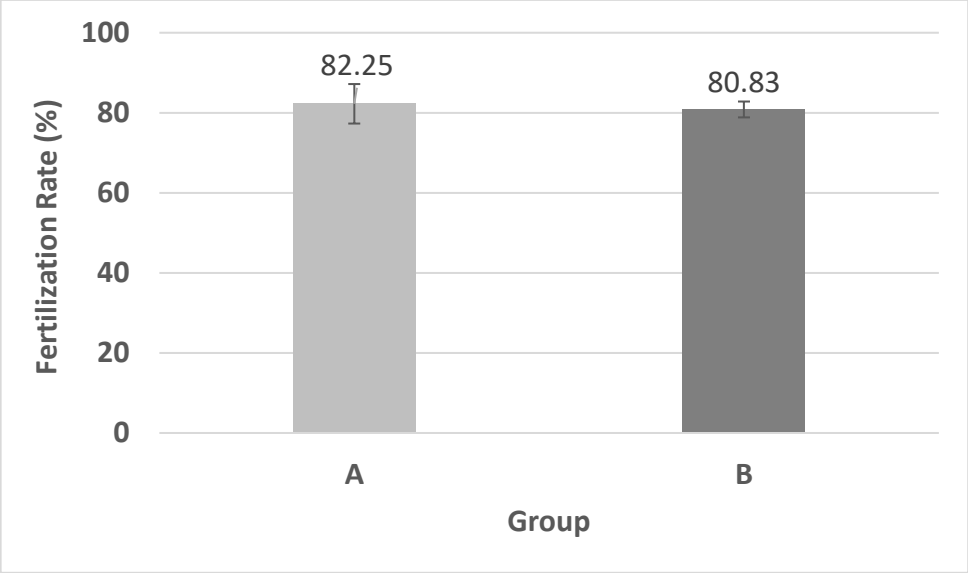

Figure 3.4: **Fertilization rates**
The fertilization rate obtained for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media), respectively. A two-sample t-test assuming equal variance (P<0.05) was performed and inferred no statistical significance between the means (p=0.5483). The error bars represent the 95% confidence interval of the sample mean.

3.2.6 Number of embryos transferred

The average number of embryos transferred per ART cycle in Group A and B are presented in Figure 3.5.

The mean number of embryos transferred in Group A and B was 1.88 ± 0.07 and 1.82 ± 0.05, respectively. A two-sample t-test with equal variance (P<0.05) indicated that there was no significant difference in the average number of embryos transferred between the two groups (p=0.2035).

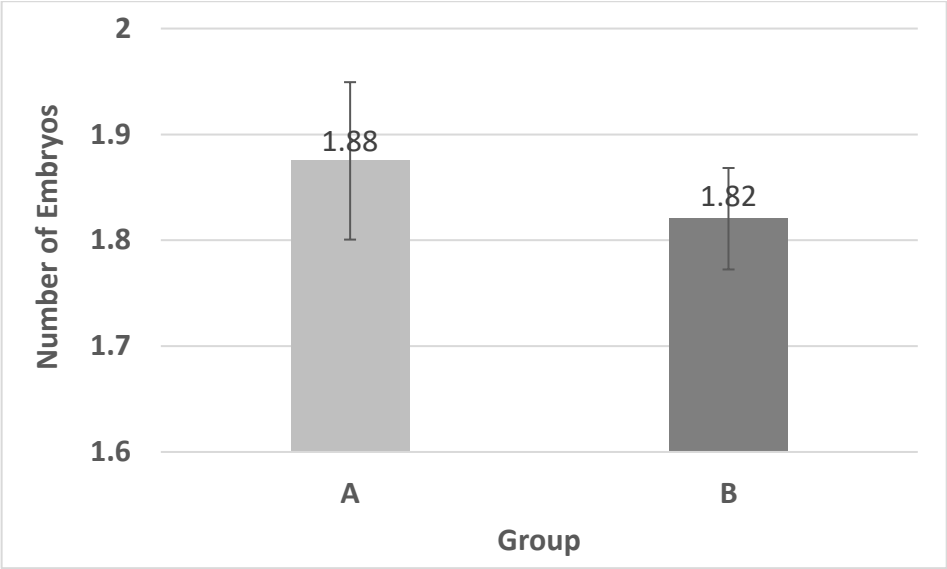

Figure 3.5: Number of embryos transferred

The number of embryos transferred refers to the average number of embryos transferred per fresh embryo transfer for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media), respectively. A two-sample t-test assuming equal variance ($P<0.05$) was performed and inferred no statistical significance between the means ($p=0.2035$). The error bars represent the 95% confidence interval of the sample mean.

3.3 Blastulation outcomes

3.3.1 Blastocyst development rates

The blastocyst development rate [BDR] (regardless of quality) and BDR (separated by quality) for Group A and B are presented in Figure 3.6(i) and Figure 3.6(ii), respectively.

In this study, the BDR refers to the proportion of normally fertilized ova [2PN] that form blastocysts (regardless of quality). In contrast, the average specific-quality BDR refers to the proportion of normally fertilized ova that form either good-quality, fair-quality, or poor-quality blastocysts.

The mean BDR for Group A was 40.70 ± 3.83 % (consisting of a good-quality BDR of 4.45 ± 1.58%, a fair-quality BDR of 17.81 ± 2.83%, and a poor-quality BDR of 18.44 ± 2.49%). In comparison, the mean BDR for Group B was 53.96 ± 2.99% (consisting of a good-quality BDR of 11.97 ± 1.82%, a fair-quality BDR of 22.27 ± 2.04%, and a poor-quality BDR of 19.80 ± 1.82%). Two-sample t-tests assuming equal variance ($P<0.05$) were performed and inferred a statistical difference between the means for the BDR (regardless of blastocyst quality) ($p<0.001$), the good-quality BDR ($p<0.001$), and the fair-quality blastocysts ($p=0.0104$). This resulted in a significant increase of the total BDR (regardless of blastocyst quality) by 13.26%, the good-quality BDR by 7.52%, and the fair-quality BDR by 4.46% for Group B when compared to Group A. However, there was no statistical difference observed between the means of poor-quality BDR ($p=0.3767$).

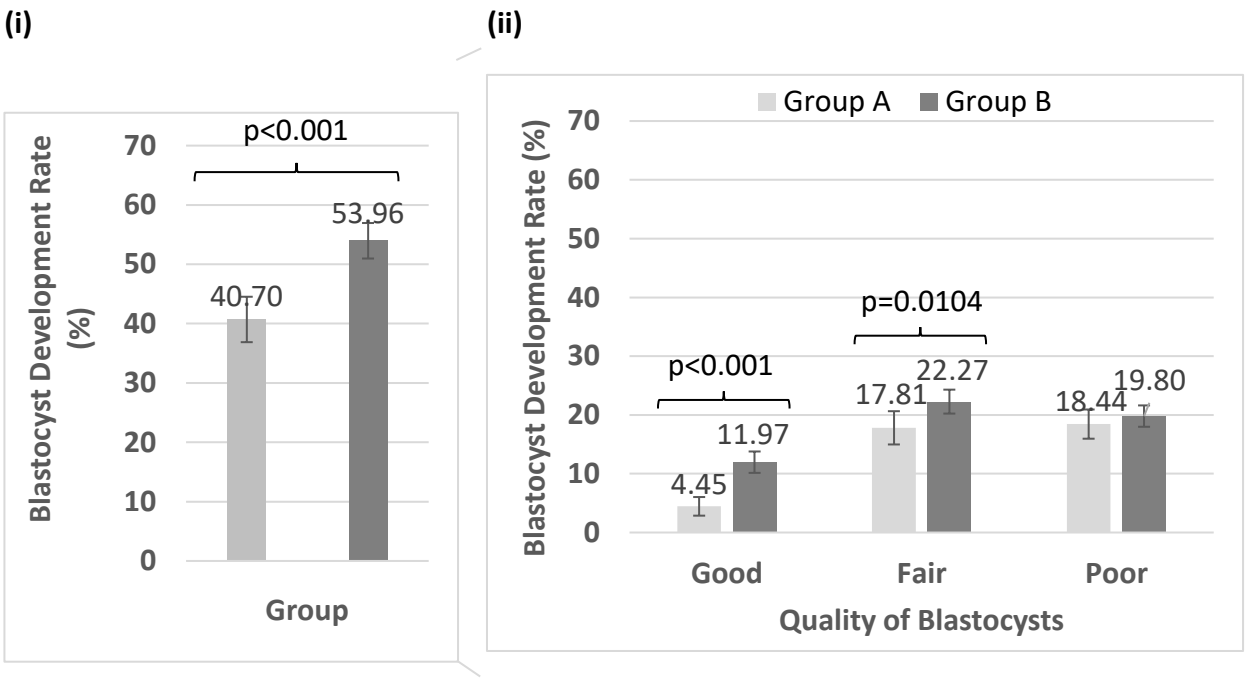

Figure 3.6: Blastocyst development rates

(i) The overall blastocyst development rate [BDR] and the **(ii)** BDR (separated by quality) obtained for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media), respectively. Two-sample t-tests assuming equal variance were performed for both the overall BDR and the BDR for each blastocyst quality (P<0.05). Statistical significance was inferred for the total BDR (p<0.001), good-quality BDR (p<0.001), and fair-quality BDR (p=0.0104) as presented on the graphs. No statistical significance was obtained for the poor-quality BDR (p=0.3767). The error bars represent the 95% confidence interval of the sample mean.

3.3.2 Proportion of various-quality blastocysts

The proportion of various-quality blastocysts obtained for Group A and B are presented in Figure 3.7.

As this analysis assesses the percentage of total blastocysts that form each quality blastocyst, only ART cycles that obtained at least one blastocyst on day 5 were considered. This resulted in the inclusion of 162/184 cycles (88.04%) ART cycles for Group A and 283/295 cycles (95.93%) ART cycles for Group B.

Taking this consideration into account, ART cycles in Group A obtained a mean percentage of 7.61 ± 2.44% good-quality blastocysts, 41.35 ± 5.23% fair-quality blastocysts, and 51.03 ± 5.61% poor-quality blastocysts. In contrast, ART cycles in Group B obtained a mean percentage of 18.92 ± 2.75% good-quality blastocysts, 41.31 ± 3.30% fair-quality blastocysts, and 39.77 ± 3.52% poor-quality blastocysts. Two-sample t-tests assuming equal variance (P<0.05) were performed and inferred a

statistical difference between the means for both good-quality blastocysts (p<0.001) and poor-quality blastocysts (p=0.005). This resulted in the significant increase of the proportion of good-quality blastocysts by 11.31% and a significant decrease of the proportion of poor-quality blastocysts by 11.26% for Group B when compared to Group A. However, there was no statistical difference observed for the proportions of fair-quality blastocysts (p=0.9873).

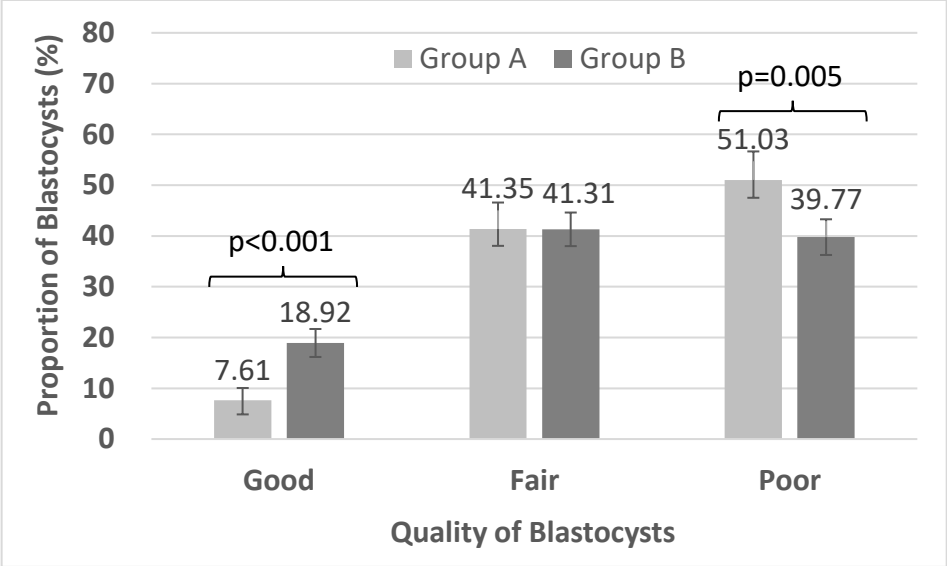

Figure 3.7: Proportion of various-quality blastocysts

The mean proportion of various-quality blastocysts obtained for Group A (n=162; in which embryos were individually cultured in sequential media) and Group B (n=283; in which embryos were co-cultured in 1-step continuous media), respectively. Two-sample t-tests assuming equal variance were performed for each quality category. Statistical significance was deduced for the good-quality blastocysts (p<0.001) and poor-quality blastocysts (p=0.005) as presented on the graph. No statistical significance was obtained for the fair-quality blastocysts (p=0.9873). The error bars represent the 95% confidence interval of the sample mean.

3.3.3 Day 5 embryo transfer rates

The day 5 embryo transfer rate for Group A and B are presented in Figure 3.8.

As defined in the Vienna consensus, the day 5 embryo transfer rate specifically refers to the proportion of cycles in which at least one blastocyst was obtained and utilized during the day 5 embryo transfer (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

The mean day 5 embryo transfer rate for Group A and B was 88.04 ± 4.72%, and 95.76 ± 2.36%, respectively. A two-sample t-test assuming equal variance (P<0.05) indicated that the mean day 5 embryo transfer rates of two groups were significantly different (p=0.0017). This inferred a significant 7.72% increase in the day 5 embryo transfer rate for Group B.

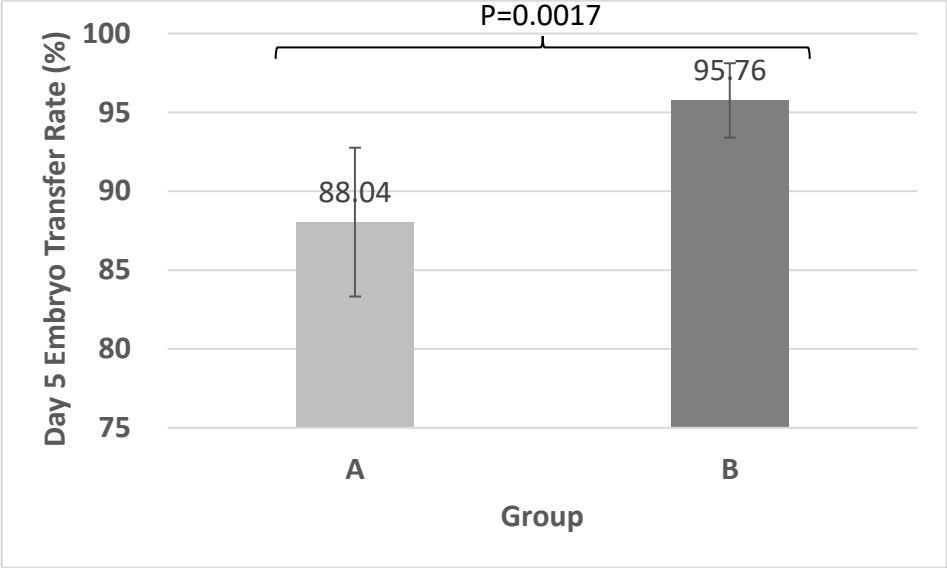

Figure 3.8: Day 5 embryo transfer rates
The mean day 5 embryo transfer rate for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media), respectively. A two-sample t-test assuming equal variance (P<0.05) inferred statistical significance between the means (p=0.0017) as presented on the graph. The error bars represent the 95% confidence interval of the sample mean.

3.3.4 Blastocyst attributions

The summary of the blastocyst attributions for Group A and B are presented in Table 3.4.

In this study, blastocyst attribution refers to a summary of the blastocysts obtained, utilized (transferred), and remained (available for cryopreservation) following the day 5 fresh embryo transfer. It should, however, be noted that this section specifically evaluates blastocyst attribution, and does not include information regarding embryos which had not yet reached this level of development by day 5 (such as cleavage stage or compacted embryos).

In terms of the average number of blastocysts obtained, Group A obtained significantly fewer blastocysts than Group B both overall and when separated into categories based on quality (3.66 ±

0.52 vs. 5.13 ± 0.47 total blastocysts, 0.48 ± 0.20 vs. 1.26 ± 0.22 good-quality blastocysts, 1.64 ± 0.31 vs. 2.02 ± 0.21 fair-quality blastocysts, and 1.54 ± 0.22 vs. 1.85 ± 0.20 poor quality blastocysts).

Regarding blastocysts transferred during day 5 of embryo culture, Group A transferred significantly fewer blastocysts than Group B (1.48 ± 0.10 vs. 1.67 ± 0.07). When comparing the quality of these blastocysts, Group A transferred significantly fewer good quality blastocysts (0.23 ± 0.07 vs. 0.66 ± 0.09), a similar number of fair quality blastocysts (0.73 ± 0.11 vs. 0.71 ± 0.09), and significantly more poor-quality blastocysts (0.53 ± 0.11 vs. 0.32 ± 0.07). This resulted in significantly fewer blastocysts post-transfer that were available for cryopreservation for Group A than Group B, both overall (2.17 ± 0.50 vs. 3.46 ± 0.46) and within each quality category (0.25 ± 0.15 vs. 0.60 ± 0.16 for good-quality blastocysts, 0.91 ± 0.28 vs. 1.32 ± 0.21 for fair-quality blastocysts, and 1.01 ± 0.21 vs. 1.54 ± 0.20 for poor quality blastocysts).

Table 3.4: Blastocyst attributions

The blastocyst attribution obtained for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media), respectively. Three categories were assessed: (i) the number (and quality) of blastocysts obtained by day 5 of embryo culture, (ii) the number (and quality) of blastocysts utilized during the day 5 embryo transfer, and (iii) the number (and quality) of blastocysts that remained and were thus available for cryopreservation. Two-sample t-tests assuming equal variance were performed between Group A and Group B for each category and blastocyst quality. In each case the values represent the mean number of blastocysts ± the 95% confidence interval. The asterisks (*) denote significant differences between the means.

Blastocyst Attribution	Group A	Group B	Probability
(i) Obtained			
Total	3.66 ± 0.52 *	5.13 ± 0.47 *	<0.001
Good-quality	0.48 ± 0.20 *	1.26 ± 0.22 *	<0.001
Fair-quality	1.64 ± 0.31 *	2.02 ± 0.21 *	0.0395
Poor-quality	1.54 ± 0.22*	1.85 ± 0.20*	0.0396
(ii) Utilized			
Total	1.48 ± 0.10 *	1.67 ± 0.07 *	0.0013
Good-quality	0.23 ± 0.07 *	0.66 ± 0.09 *	<0.001
Fair-quality	0.73 ± 0.11	0.71 ± 0.09	0.7430
Poor-quality	0.53 ± 0.11 *	0.32 ± 0.07 *	0.0008
(iii) Remained			
Total	2.17 ± 0.50 *	3.46 ± 0.46 *	0.0003
Good-quality	0.25 ± 0.15 *	0.60 ± 0.16 *	0.0038
Fair-quality	0.91 ± 0.28*	1.32 ± 0.21 *	0.0207
Poor-quality	1.01 ± 0.21 *	1.54 ± 0.20 *	0.0009

3.4 ART outcomes

3.4.1 Implantation rates

The average implantation rate for the embryos transferred in Group A and B are presented in Figure 3.9.

The mean implantation rate for Group A and Group B was 36.23 ± 6.16%, and 38.36 ± 4.49%, respectively. A two-sample t-test with equal variance (P<0.05) indicated that there was no significant difference in the implantation rates between the two groups (p=0.5762).

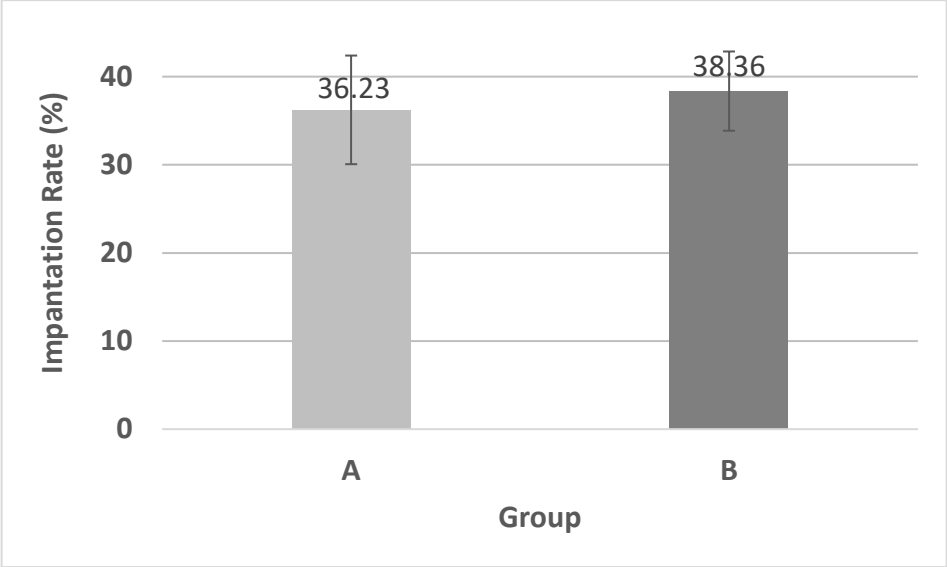

Figure 3.9: Implantation rates
The mean implantation rate for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media), respectively. A two-sample t-test assuming equal variance (P<0.05) inferred no statistical significance between the means (p=0.5762). The error bars represent the 95% confidence interval of the sample mean.

3.4.2 Clinical pregnancy rates

The average clinical pregnancy rate for Group A and B are presented in Figure 3.10.

The mean clinical pregnancy rate for Group A and Group B was 46.74 ± 7.25% and 54.92 ± 5.71%, respectively. A two-sample t-test with equal variance (P<0.05) indicated that there was no significant difference in the clinical pregnancy rates between the two groups (p=0.0819).

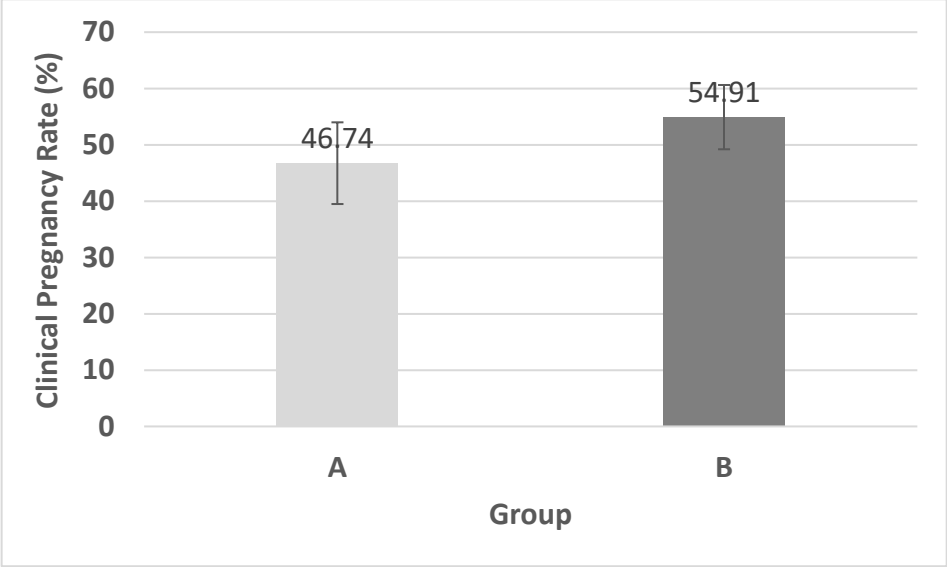

Figure 3.10: Clinical pregnancy rates
The mean clinical pregnancy rate for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media), respectively. A two-sample t-test assuming equal variance (P<0.05) inferred no statistical significance between the means (p=0.0819). The error bars represent the 95% confidence interval of the sample mean.

3.4.3 Live birth rates

The average live birth rate for Group A and B are presented in Figure 3.11.

The mean live birth rate for Group A and Group B was 41.85 ± 7.17% and 47.12 ± 5.73%, respectively. A two-sample t-test with equal variance (P<0.05) indicated that there was no significant difference in the live birth rates between the two groups (p=0.2604).

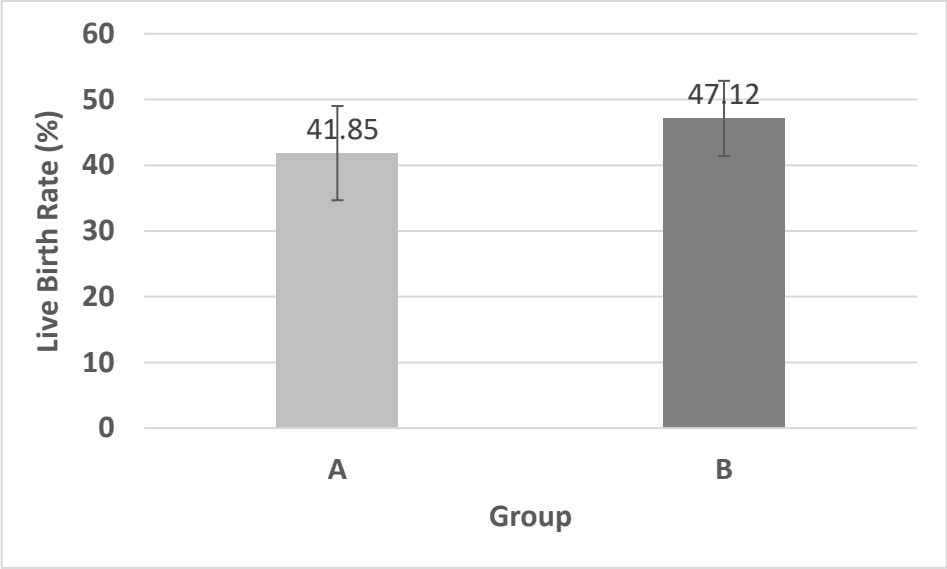

Figure 3.11: Live birth rates
The mean live birth rate for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media), respectively. A two-sample t-test assuming equal variance (P<0.05) inferred no statistical significance between the means (p=0.6723). The error bars represent the 95% confidence interval of the sample mean.

3.4.4 Miscarriage rates

The average miscarriage rate for Group A and B are presented in Figure 3.12.

The mean live birth rate for Group A and Group B was 10.33 ± 4.42% and 9.15 ± 3.31%, respectively. A two-sample t-test with equal variance (P<0.05) indicated that there was no significant difference in the miscarriage rates between the two groups (p=0.6723).

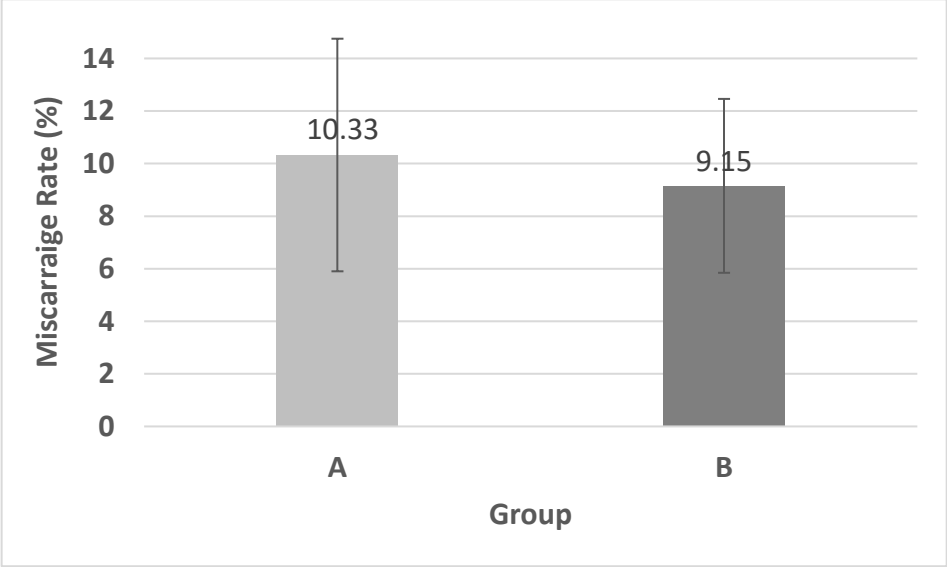

Figure 3.12: Miscarriage rates
The mean miscarriage rate for Group A (n=184; in which embryos were individually cultured in sequential media) and Group B (n=295; in which embryos were co-cultured in 1-step continuous media), respectively. A two-sample t-test assuming equal variance (P<0.05) inferred no statistical significance between the means (p=0.2604). The error bars represent the 95% confidence interval of the sample mean.

CHAPTER 4

DISCUSSION

Over the last few decades, advancements in assisted reproductive technology [ART] have significantly improved infertility treatments (Thouas and Gardner, 2010). One of the research areas, which has received a lot of attention, is the method of embryo culture (Thouas and Gardner, 2010). Several researchers have reported modifications that can improve blastulation and ART outcomes (Ebner *et al*., 2010; Thouas and Gardner, 2010). Needless-to-say, well-documented modifications that were consistently reported to improve embryo culture have been globally accepted and incorporated into the fertility clinics' standard operating procedures [SOPs]. In contrast, adaptations with limited or contradictory results are still under review. In these cases, fertility clinics are recommended to perform their own internal studies to determine whether these modifications improve their clinical outcomes based on their patient cohort and laboratory limitations (Sfontouris *et al*., 2016; Morbeck *et al*., 2017; Deng *et al*., 2020).

4. 1 Study Outcomes

This study aimed to evaluate the effects of changing the embryo culture method at Drs. Aevitas Fertility Clinic from Embryo Culture Method A (where embryos were cultured individually in sequential media) to Embryo Culture Method B (where embryos were co-cultured in continuous media). This was performed by initially investigating the blastulation outcomes (objective 1) and ART outcomes (objective 2) of embryos cultured using the two embryo culture methods and later determining whether this study supports the continued use of Embryo Culture Method 2 for future ART cycles (objective 3).

In summary, the study outcomes indicated that Group B obtained statically better blastulation outcomes than Group A. Although the ART results for the two groups were not significantly different, Group B also tended to obtain slightly better ART outcomes than Group A. These results, together with the practical and economic advantages of co-culturing embryos in continuous media, supported the continued use of Embryo Culture Method B for future ART cycles at Drs. Aevitas Fertility Clinic.

4.1.1 Blastulation outcomes

The **primary objective** of this study was to determine the effects of changing the embryo culture system on the **blastulation outcomes** at Drs. Aevitas Fertility Clinic.

A. Blastulation results

Compared to Group A, Group B was shown to obtain superior blastulation outcomes, resulting in significantly: higher **blastocyst development rates [BDRs]**, a higher **proportion of better-quality blastocysts**, an improved **day 5 embryo transfer rate**, and a more advantageous **blastocyst attribution profile**.

More specifically, the change in the culturing method, from Embryo Culture Method A to B, resulted in a significant improvement in the **total BDR** by 13.26% (40.70% to 53.96%). Although both culture systems retained values above the recommended competency value (≥40%), the significant improvement in the total BDR of Group B suggested that co-culturing embryos in continuous media were better at supporting embryo kinetics (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

In addition, when investigating **specific-quality BDRs**, Embryo Culture Method B resulted in significantly improved BDRs for the better-quality blastocysts [good-quality BDR (11.97% vs. 4.45%) and fair-quality BDR (22.27% vs. 17.81%)]. Most importantly was the doubling of the **good-quality BDR** for Group B. Although both these values are lower than that recommended for competency by the Vienna consensus (≥30%), it should be remembered that this is an objective parameter (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). As such, both the strictness of embryo grading and this study's choice to report blastulation results for day 5 only instead of day 5 and 6 is expected to affect these values (Richardson *et al.*, 2015; ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Regardless, the results obtained suggest that the change in the culture method significantly improved the number of good-quality blastocysts, which positively correlates to the blastocysts' prognostic potential to implant, achieve a clinical pregnancy, and result in a live birth (Schoolcraft *et al*., 1999; Alfarawati *et al*., 2011; Richardson *et al*., 2015; Zhao *et al*., 2018). Not only can this improve the ART results following fresh embryo transfer, but the higher number of good-quality blastocysts is hypothesized to improve cumulative ART outcomes derived from a single cohort of oocytes (Sfontouris *et al*., 2016).

The above results are re-iterated in the **proportion of various quality embryos**. This performance indicator deduced that Group B significantly improved the **proportion of better-quality blastocysts** [with more good-quality (18.92% vs. 7.61%) and fewer poor-quality blastocysts (39.77% vs. 51.03%)]. These results indicated that Group B was more effective at consistently supporting the development of good-quality blastocysts (Richardson *et al.*, 2015).

On the other hand, a significantly improved **day 5 embryo transfer rate** (95.76% vs. 88.04%) was reported for Group B and resulted in 7.72% more patients in this group obtaining at least one blastocyst to transfer. Although this performance indicator does not consider the blastocyst quality, it does emphasize the culture system's ability to support developing embryos and allow for the transfer of at least one day 5 blastocyst to continue as planned (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Furthermore, there is an increased likelihood of implantation as the uterine lining's receptivity and the embryo's readiness to implant are expected to be better aligned (Richardson *et al.*, 2015).

Lastly, in addition to these standardized blastulation performance indicators, this study also investigated the average **blastocyst attribution profile** for Group A and B (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). These profiles retain valuable information regarding the mean number and quality of blastocysts obtained, utilized, and available cryopreservation. As such, the analysis of these profiles can contribute meaningful insight into the potential cumulative ART outcomes derived from the average cohort of oocytes (Sfontouris *et al*., 2016).

In this study, the profiles indicated that the mean number of **blastocysts obtained** for Group B was significantly higher than Group A and resulted in an average of 1.47 more blastocysts per patient. Furthermore, there were significantly more blastocysts within each quality category. This was especially notable for good-quality blastocysts, in which the average number of blastocysts available to Group B was more than double that of Group A (1.26 vs. 0.48 embryos per ART cycle).

The increased number and better quality of blastocysts obtained in Group B were further reflected in the number of blastocysts available following embryo culture.

First, regarding **blastocysts utilized**, although the two groups transferred a similar number of embryos (Section 3.2.6), Group B was shown to transfer significantly more, and of better-quality, blastocysts. Although it should be remembered to differentiate between the formation and viability

of blastocysts, multiple studies have reported a strong correlation between the utilization of better-quality blastocysts to improved embryos prognostic potential to implant, achieve a clinical pregnancy, and result in a live birth (Richardson *et al*., 2015; Sfontouris *et al*., 2016; ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). As such, it can be inferred that Group B utilized blastocysts that retained a higher probability of achieving preferable ART outcomes following the fresh embryo transfers.

Second, Group B retained an average of 1.29 more blastocysts available for cryopreservation regarding the number and quality of the blastocysts remaining. Furthermore, there were significantly more embryos in each quality category. Interestingly, not only was the number of good-quality blastocysts available to Group B more than double that for Group A (0.60 vs. 0.25), but this value was even higher than the average number of good-quality blastocysts initially obtained in Group A before embryo transfer (0.60 vs. 0.48). Thus, based on the quality of the embryos available for cryopreservation, Group B is hypothesized to retain significantly better cumulative ART outcomes than Group A (Sfontouris *et al*., 2016).

B. Blastulation outcomes in the context of previously published literature

This study hypothesized that co-culturing embryos in continuous media would statistically improve the blastulation outcomes in Group B (Chapter 1, hypotheses). Although it should be noted that no studies investigated the change in both these factors (co-culture and continuous media) co-currently, there were also several reports which support either the use of co-culturing human embryos (opposed to individual culture) or the utilization of continuous media (opposed to sequential media) to obtain beneficial blastulation outcomes, as indicated below (Donmez *et al*., 2008; Sepúlveda *et al*., 2009; Ebner *et al*., 2010; Rebollar-Lazaro and Matson, 2010; Khoury *et al*., 2012; De la Calle *et al*., 2013; Tao *et al.*, 2013; Alteri *et al*., 2015; Sfontouris *et al*., 2014; Sfontouris *et al*., 2016).

Donmez *et al*. (2008) performed a randomized sibling study to compare the optimal culture medium (sequential vs. one-step) on embryonic development and blastocyst formation in IVF. They reported a significant increase in signs of embryo compaction on day 3 and blastocyst development on day 5 using single-step media ($p<0.05$).

Khoury *et al*. (2012) also performed a randomized sibling study and reported an improvement in blastocyst development using a single-step medium versus sequential medium. These researchers

found a significant increase in the blastocyst formation rate on day 5 (63% vs. 54%). Furthermore, they indicated borderline significance in both the total number of blastocysts available for transfer and the total number of blastocysts available for cryopreservation, following embryo culture in a single-step medium (46% vs. 39%).

Similar findings were reported by **De la Calle *et al.* (2013)**. Who recorded a significant increase in the total BDR and blastocyst utilization rates when utilizing single-step media (51.5% vs. 40.7%).

Whereas **Sepúlveda *et al*. (2009)** went on to investigate the embryos' readiness to implant together with the more generally examined total BDRs. They reported a significant improvement in both the day 5 blastulation rate (42.9% vs. 31.1%) and the percentage of either partially or fully hatched blastocysts at the time of transfer (24.7% vs. 13.7%). In conclusion, these researchers suggested that continuous media did not only improve *in vitro* development but ensured the possession of important morphologic attributes needed to implant.

Lastly, similar findings related to the total BDR were presented by **Sfontouris *et al*. (2016)**, who published a systematic review and meta-analysis of randomized controlled studies investigating the blastocyst culture using single versus sequential media in clinical of 23 studies. These authors concluded that although both media were adequate to support developing embryos, single media was associated with increased blastocyst formation per randomized oocyte or zygote. However, although they further reported that studies such as **Alteri *et al*. (2015)** and **Sfontouris *et al*. (2014)** went on to infer that single-step culture medium is associated with better blastocyst quality, there was insufficient evidence to confirm this association due to limited studies, and inadequate sample sizes, reporting similar results.

In studies comparing the embryo culture method employed, **Rebollar-Lazaro and Matson (2010)** studied the effects of group culture of cleavage-stage embryos from day 1-3. While this study reported that the implantation and ongoing pregnancy rates were similar for embryos either individually or co-cultured during early embryo culture, blastulation utilization rates were significantly better for co-cultured embryos (51.3% versus 46.5%) in women <35 years. This resulted in significantly more embryos available for both transfer and cryopreservation.

Whereas **Ebner *et al*. (2010)** performed a prospective sibling study comparing individual and group culture from day 3 of culture until blastulation. These researchers reported significantly better

blastulation outcomes for co-cultured embryos. More specifically, co-cultured embryos attained significantly better total and top-quality blastocytes rates than those cultured individually ($p<0.05$).

Lastly, **Tao *et al.* (2013)** also studied the effect of co-culturing human embryos from day 3. However, these researchers also considered the quality of embryos co-cultured together. They concluded that the embryos' quality should be considered, and embryo categories potentially separated to improve total BDRs (following these results: 72.4% for good-quality embryos co-cultured together, 44.0% for mixed-quality embryos co-cultured together, and 23.0% for poor-quality embryos co-cultured together).

Although it should be remembered that for both the type of media utilized and method of culturing embryos employed, other studies report contradictory results (Almagor *et al*., 1996; Spyropoulou *et al*., 1999; Macklon, 2002; Ciray, 2012; Patrick, 2013; Sfontouris, 2015; Werner *et al*., 2016), the previously published literature presented above, are in support of the results obtained in this study. These papers re-iterate the following two potential reasons (or a combination thereof) for the improvement in the blastulation outcomes in this study.

The first is the utilization of continuous culture media, where the nutritional requirements for cleavage, compaction, and blastulation is delicately balanced (Donmez *et al*., 2008; Sepúlveda *et al*., 2009; Khoury *et al*., 2012; De la Calle *et al*., 2013; Sfontouris *et al*., 2016). As such, embryos can choose when and which nutrients to utilize at any given development stage. Furthermore, it is hypothesized that by including all 20 amino acids from day one, embryo kinetics is improved by activating gene transcription earlier and resulting in the faster compaction and blastulation on day 4 and 5, respectively (Sepúlveda *et al*., 2009; De la Calle *et al.*, 2013). This is an important consideration, as day 5 marks an important milestone for the embryos and is the time in which blastulation outcomes are determined (Donmez *et al*., 2008; Sepúlveda *et al*., 2009; Khoury *et al*., 2012; De la Calle *et al*., 2013; Sfontouris *et al*., 2016). In addition, it should be recognized that, the utilization of sequential media requires a media change on day 3. This is hypothesized to possibly reduce the embryos' kinetics, temporarily, as the embryos overcome the shock of transferring embryos from one media to the other (Sfontouris *et al*., 2016; Morbeck *et al*., 2017; Deng *et al*., 2020).

The second potential reason for the improvement in the blastulation outcomes of Embryo Culture System B in the current study, is the co-culturing of the embryos (Ebner *et al*., 2010; Rebollar-Lazaro and Matson, 2010; Tao *et al.,* 2013). It is well-established that the optimal growing environment for

embryos requires more than a balance in nutrition (Reed *et al*., 2011; Tao *et al.,* 2013). As such, it is important to consider the interactions between a developing embryo and its natural surroundings *in vivo.* Within the female's reproductive tract, an embryo is exposed to several autocrine (targets own cells), paracrine (targets nearby cells), and endocrine (targets the circulatory system) mediators (Ebner *et al*., 2010). In individual culture, these intercellular interactions with surrounding tissues do not exist. However, when co-cultured (at a high enough density), surrounding embryos create localized zones of accumulated embryo-trophic factors which positively affect the growth and development of the surrounding embryos (Ebner *et al*., 2010; Rebollar-Lazaro and Matson, 2010; Tao *et al*., 2013; Kelley and Gardner, 2017). For instance, factors such as Insulin-like growth factor I and II have been reported to help regulate cell growth, proliferation, and embryo survival; and Platelet-activating factor has been reported to reduce apoptosis and increase mitosis, ultimately improving the BDR and day 5 embryo transfer rate (Ebner *et al*., 2010; Agrogiannis *et al*., 2014). Furthermore, growth factors and chemokines secreted by top-quality embryos have been shown to enhance the differentiation and decidualization to improve the good-quality BDRs (Tao *et al.,* 2013; Sadeghi, 2017).

However, regardless of the specific reason for the improved blastulation outcomes (or a combination thereof), this study indicated that changing the culture method from Embryo Culture Method A to B significantly improved the blastulation outcomes Drs. Aevitas Fertility Clinic.

4.1.2 ART outcomes

The **secondary objective** of this study was to determine the effects of changing the embryo culture system on the **ART outcomes** at Drs. Aevitas Fertility Clinic.

A. ART results

In this study, Group B was shown to retain favourable mean values for all the ART categories, resulting in higher **implantation rates** (38.36% vs. 36.23%), higher **clinical pregnancy rates** (54.91% vs. 46.74%, 8.17% difference), lower **miscarriage rates** (9.15% vs. 10.33%), and higher **live birth rates** (47.12% vs. 41.85%, 5.27% difference). However, no statistical significance was reported between the two groups' means for any ART outcomes. Thus, these results indicate that while Group B may retain slightly improved ART outcomes than Group A, there was insufficient evidence to either support or refute the use of either embryo culture method based on the ART outcomes alone.

B. ART outcomes in the context of previously published literature

This study hypothesized that a statistically significant increase in the blastulation outcomes (indicated by the production and utilization of more and better-quality day 5 blastocysts in Group B) would significantly increase the down-stream ART outcomes obtained from the fresh embryo transfer cycles (Chapter 1, hypotheses). This hypothesis was based on the findings that superior embryo quality is positively associated with ART outcomes (Schoolcraft *et al*., 1999; Alfarawati *et al*., 2011; Richardson *et al*., 2015; Zhao *et al*., 2018).

However, while a trend of improved mean values for each ART outcome in Group B, statistical significance was not attained. Although initially surprising, previously published literature have reported similar findings, in which the use of continuous embryo culture media (opposed to sequential media) or the employment of embryo co-culture (opposed to individual culture) resulted in significantly improved blastulation outcomes but did not retain statically significant increases in any or all ART outcomes measured (Sepúlveda *et al*. 2009; Ebner *et al*. 2010; Rebollar-Lazaro and Matson, 2010; Khoury *et al*., 2012; Scarica *et al*., 2015; Sfontouris *et al*., 2016; Werner *et al*., 2016; Cimadomo *et al*., 2018; Deng *et al.*, 2020).

In studies comparing embryo media utilization: **Sepúlveda *et al*. (2009)** reported a significant increase in the day 5 blastocyst development rate of embryos cultured in continuous media when compared to those cultured sequential media (42.9% vs. 31.1%). Although the embryos cultured in continuous media sustained higher implantation rates and a positive trend for both the clinical

pregnancy rate and ongoing pregnancy rate, statistical significance was not inferred for the latter two ART outcomes.

Similar outcomes were obtained by **Khoury *et al*. (2012)**, who performed a sibling study on embryos cultured in either continuous or sequential media. Their results indicated that embryos cultured in continuous media retained a significantly higher day 5 blastocyst formation (63% vs. 54%) and enabled significantly more blastocysts to be transferred per fresh embryo transfer (1.53 vs. 1.38). However, although both implantation rates and clinical pregnancy rates were slightly improved, no statistical significance was deduced between that of the two media utilized.

Deng *et al.* (2020), on the other hand, reported a significantly higher total blastulation formation rate for embryos cultured in a SAGE 1-step continuous medium as opposed to a SAGE advantage sequential medium (51.7% vs. 43.3%) and similar clinical pregnancy rates, miscarriage rates and live birth rates between the two culture systems. However, these researchers went on to investigate the ploidy rate of the blastocysts obtained. They reported a significantly higher aneuploidy rate for embryos cultured in the continuous media (54.0% vs. 45.8%) and went on to suggested that this may be the reason for their lack of improved ART outcomes regardless of superior blastulation outcomes obtained.

However, in other studies with a similar significant increase in blastulation outcomes for embryos cultured in continuous media, **Werner *et al*. (2016)** reported a higher percentage of usable euploid embryos obtained from embryos cultured in sequential media, and **Scarica *et al*. (2015)** and **Cimadomo *et al*. (2018)** reported comparable euploidy rates for embryos cultured in both mediums.

Finally, similar findings were concluded by **Sfontouris *et al*. (2016)**, who published a systematic review and meta-analysis of randomized controlled studies investigating the blastocyst culture using single versus sequential media in clinical of 23 studies. These authors concluded that although a single medium for extended culture appeared to result in higher blastocyst formation, there was insufficient evidence to recommend the use of single-step media, based on the ART results.

In addition, similar results were reported in previous literature whereby significantly improved blastulation outcomes were retained following co-culturing a density of approximately 1-5 embryo/50 µl (utilized in this study) but did not result in statically significant increases in any or all ART outcomes measured.

For instance, **Rebollar-Lazaro and Matson (2010)** studied the effects of co-culturing human embryos in groups of 3 or more in 15 µl of media. They reported that the blastocyst utilization rate on day 5 of embryo culture was significantly higher for co-cultured embryos than individually cultured (51.3% versus 46.5%) in women <35 years old. However, similar implantation rates, clinical pregnancy rates, and ongoing pregnancy rates were obtained.

Whereas a study by **Ebner *et al*. (2010)** studied the effects of co-culturing human embryos in groups of 1-5 embryos per 30 µl of media up until day 5. These researchers reported significantly superior blastulation rates (55.8% vs. 45.2%) and percentage of top-quality blastocysts (79.2% vs. 64.7%) from co-cultured embryos when compared to individually cultured embryos. Following elective fresh embryo transfers, however, the implantation rates and pregnancy outcomes were similar. Whereas, when considering frozen embryo transfers (in the case of delayed transfer in patients presenting with signs of ovarian hyperstimulation), there was a non-significant tendency for higher clinical pregnancy rates and live birth rates from co-cultured embryos than those individually cultured.

These previously published studies indicate that the results obtained in the current study are similar to and supported by previously published literature. In conclusion, this study's results suggest one of two possibilities.

First, there may be a slight advantage to the combination of co-culturing embryos in continuous media; however, the power of the statistical tests performed was insufficient to deduce significance between the means (Kim, 2015). This can occur when the population's sample size is too small to overcome data distribution (Kim, 2015). This is a possibility in this study as only included 479 cycles (separated into 184 cycles in Group A and 295 cycles in Group B) (Section 3.1). Furthermore, although blastulation outcomes (such as the total BDR and the proportion of good-quality blastocysts) were calculated from several normally fertilized zygotes and blastocysts obtained per ART cycle. ART outcomes (such as the clinical pregnancy and live birth rate) are represented by an extremity of 0% or 100% (0% representing the absence of a clinical pregnancy or live birth, or 100% representing the presence of a clinical pregnancy or live birth). As such, the sample means for blastulation outcomes are usually closer to that of the population mean when compared to the ART outcomes.

This is an important consideration as standard error, standard deviation, confidence intervals, and statistical differences are calculated based on the difference between the sample means and the

population mean and sample size (Kim, 2015). As such, by its very nature, ART outcomes will result in larger error bars and require a larger sample population to deduce significance than that for blastulation outcomes.

Secondly, there is no difference in the ART outcomes. Thus, although significantly more and better-quality embryos were transferred in Group B than Group A, this did not translate to improved ART outcomes due to unknown factors (Sfontouris *et al*., 2016; Deng *et al.*, 2020). For instance, in the case of **Deng *et al*. (2020)**, researchers reported that although embryos cultured in continuous media obtained higher blastulation rates and better morphological grades than those cultured in sequential media, there was an increase in aneuploidy rate that resulted in similar ART outcomes between the two groups (Deng *et al.*, 2020). This could occur from the difference in media formulation and replacement schedules that affect the pH, osmolarity, and build-up of harmful by-products and can go on to impact embryo development (Deng *et al*., 2020).

Furthermore, although the aneuploidy status of an embryo is a valuable indicator of an embryo's potential to implant and result in a live birth (Alfarawati *et al*., 2011; Zhao *et al*., 2018), this confounding factor (aneuploidy) was not considered in our study. Most patients at Drs. Aevitas Fertility Clinic does not choose to utilize pre-genetic implantation testing for aneuploidy [PGT-A]. This, however, is not unusual based on laboratory limitations, costs involved, or positive association between morphological grades with ART outcomes (Sepúlveda *et al*. 2009; Ebner *et al*. 2010; Rebollar-Lazaro and Matson, 2010; Alfarawati *et al*., 2011; Khoury *et al*., 2012; Scarica *et al*., 2015; Sfontouris *et al*., 2016; Zhao *et al*., 2018). Therefore, it is important to remember that there is a weak correlation between morphological grades and the aneuploidy status of embryos (aneuploid embryos can achieve the highest morphologic scores, and euploid embryos can present with poor morphologies). Thus, this difference in morphological grades and aneuploidy rates should be considered when utilizing two different culture mediums (Alfarawati *et al*., 2011).

Regardless of the reason for the similar ART outcomes in our study, however, it can be concluded that in both these cases, there are no detrimental effects on ART outcome in changing the culture method from Embryo Culture Method A to Embryo Culture Method B.

4.1.3 Evaluation of the embryo culture methods

The **tertiary and final objective** of this study was to evaluate the embryo culture methods utilized to determine whether this study's findings support the continued use of Embryo Culture Method B at Drs. Aevitas Fertility Clinic in future cycles. As such, both the blastulation and ART outcomes obtained, together with the additional advantages of co-culturing embryos in a continuous medium, was considered.

Firstly, this study considered the utilization of continuous culture media on the **blastulation outcomes**. The results indicated a significant improvement in the total blastocyst development rate, good-quality blast blastocyst development rate, proportion of good-quality blastocysts, and day 5 embryo transfer rate. This concluded that the average patient whose embryos were co-cultured in continuous media obtained significantly more and better-quality blastocysts than the average patient whose embryos were individually cultured in sequential media. This deduced that more morphologically superior blastocysts could be transferred and cryopreserved for future use for this cohort of patients.

Secondly, this study considered the **ART outcomes** retained. The results indicated that embryos co-cultured in continuous media retained slightly improved ART outcomes (higher implantation rates, higher clinical pregnancy rates, lower miscarriage rates, and higher live birth rates) than embryos individually cultured in sequential media. Although these ART outcomes were not statistically significant and thus provided insufficient evidence to either support or refute the use of either embryo culture method based on these findings alone, it extrapolated that there were no detrimental effects on the ART outcomes identified from the use of Embryo Culture Method B.

Thirdly, this study considered several **additional advantages** of co-culturing embryos in continuous media.

It acknowledged that, first, **economically**, co-culturing several embryos per microdroplet reduce the number of culture drops required per patient (Reed *et al*., 2011). This is especially important in laboratories such as Drs. Aevitas Fertility Clinic, several patients routinely obtained more than 8-10 ova per aspiration (Sfontouris *et al*., 2016; Deng *et al*., 2020). Furthermore, the use of continuous culture (omitting a day 3 change in media) also reduces both the costs related to quality testing on day 3 and laboratory costs by decreasing the number of consumables utilized (such as culture media, culture dishes, and glass pipettes) (Sfontouris *et al*., 2016; Werner *et al*. 2016; Deng *et al*., 2020).

Second, **practically**, the use of continuous media reduces labour intensity within the ART laboratory in both the preparation of culture dishes and the transfer of embryos on day 3 (Sfontouris *et al*., 2016; Werner *et al*., 2016; Deng *et al*., 2020). In addition, it reduces the number of dishes being equilibrated or utilized at any given time (Werner *et al*., 2016). This, again, is useful in laboratories such as Drs. Aevitas Fertility Clinic, in which embryologists have limited time and space to perform to prepare culture dishes and transfer embryos, and space within the incubators is limited (Sfontouris *et al*., 2016; Werner *et al*., 2016). Moreover, in laboratories utilizing time-lapse technology, continuous media retains an added benefit in that embryos do not need to be removed from the machines, which results in missing imagining on day 3 (Sfontouris *et al*., 2016).

Third, from a **risk management** perspective, the addition of steps to any standard operating procedure introduces risk. Thus, the utilization of continuous media (without a media change on day 3) lowers the embryo culturing method's risk profile by reducing unintentional handling errors (Deng *et al*., 2020).

Thus, taking the blastulation outcomes, ART outcomes, and additional advantages of co-culturing embryos in a continuous media into account, this study **supports the continued use of Embryo Culture Method B at Drs' Aevitas Fertility Clinic in future cycles**.

4.2 Confounding factors

Potential confounding factors are routinely considered to ensure that study groups are balanced and comparable (Skelly *et al*., 2012). In doing so, the outcomes obtained can be used to ensure whether the study objectives can be evaluated without the risk of introducing population bias and generating incorrect associations (Mann, 1995; Skelly *et al*., 2012).

In this study, the following seven confounding factors were investigated: (1) male and female diagnoses, (2) assisted reproductive technique employed, (3) ova age (female age), (4) number and maturity of ova obtained from aspiration, (5) fertilization rate, (6) day of embryo transfer, and (7) the number of embryos transferred.

In summary, the results obtained indicated that these factors were similar for both groups, with no statical significances identified. Thus, these factors were shown to not impact the outcomes of this study.

4.2.1 Male and female diagnoses

Male and female diagnoses identify physiological irregularities within the patient cohort (Gardener *et al*., 2008). Each diagnosis contains its own set of challenges, with which ART can be utilized to either completely overcome or alleviate the known causes of infertility (Gardener *et al*., 2008; Ozkan *et al*., 2008; Child, 2013; Kruger, 2016; Hojnik and Kovačič, 2019). As such, the couple's prognosis for ART success is indeed dependent on the patient diagnoses and the ART method utilized. The diagnostic profiles for Group A and B were thus considered and assessed as a potential confounding factor of this study. However, this study indicated that the male and female diagnostic profiles for the two groups were not statistically different.

The most prevalent diagnoses for men, within the cohort, were normozoospermia (65.22% vs. 66.10%), oligozoospermia (9.78% vs. 11.19%), azoospermia (for whom testicular spermatozoa were utilized) (10.33% vs. 7.80%), and teratozoospermia (7.07% vs. 7.12%). Whereas the most prevalent diagnoses for women were ovarian insufficiency (32.61% vs. 40.68%), normal fertility parameters (40.23% vs. 31.19%), endometriosis (9.24% vs. 8.14%), and anatomical factors (8.15% vs. 9.15%). These results indicated that the patient cohort presented with a variety of both normal fertility parameters (such as normozoospermia) as well as identifiable infertility parameters, which could either be completely overcome using an appropriate ART (such as oligozoospermia, anatomical

factors were the oviducts are blocked or absent, or ovarian insufficiency when utilizing an ova donor) and those for which infertility factors could be alleviated but not directly overcome with the use of ART (such as endometriosis or ovarian insufficiency when utilizing autologous ova) (Gardener *et al*., 2008; Ozkan *et al*., 2008; Child, 2013; Kruger, 2016; Hojnik and Kovačič, 2019).

4.2.2. Assisted reproductive technique employed

The assisted reproductive technique [ART] employed refers to the method of ova insemination utilized (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). There were two main ART categories in this study: in vitro fertilization [IVF] and intracytoplasmic sperm injection [ICSI] techniques. Each method has its advantages, and employment was carefully chosen for each cycle based on the couple's diagnoses (Hojnik and Kovačič, 2019; Liu and Baker, 2000). Despite this, it should be remembered that that choice of category employment is known to influence the cycle outcomes (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017; Hojnik and Kovačič, 2019). For instance, where ICSI techniques are expected to improve the fertilization rate, which can, in turn, affect the blastulation outcomes by increasing the number of zygotes with the potential to go on to form blastocysts (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017; Lee *et al*., 2017). IVF is reported to improve the overall ART outcomes by increasing the clinical pregnancy and live birth rate of those blastocysts transferred (Eftekhar *et al*., 2012). Therefore, ART utilization profiles for Group A and B were considered and assessed as a potential confounding factor in this study.

However, this study indicated that a similar percentage of each ART was utilized in both groups, with most cycles employing ICSI only techniques (77.72% vs. 82.71%), fewer cycles utilizing a combination of both IVF and ICSI cycles (20.65% vs. 16.27%), and the minority of cycles employing IVF only (1.63% vs. 1.02%). Furthermore, the statistical analysis indicated that these profiles were not statistically different.

4.2.3. Ova age (female age at the time of oocyte retrieval)

Ova age, or the female's age at the time of oocyte retrieval, is another well-reported confounding factor of ART success (Cimadomo *et al*., 2018). Several studies have reported a strong correlation between a woman's age and the average number and quality of ova obtained at aspiration (Lawler *et al.*, 2007; Liu and Case, 2011; Fleming *et al*., 2015). As the ova age increases, not only is

there a reduction in the number of ova and thus potential blastocysts available for transfer (Sauer *et al.*, 1990; Lawler *et al.* 2007; Liu and Case, 2011; Fleming *et al*., 2015; Capalbo *et al.*, 2017; La Marca *et al.*, 2017; Thomas *et al*., 2010). But the reduction in ova quality decreases the blastocyst development rate, the proportion of good quality blastocysts, the day 5 embryo transfer rate, and several ART outcomes (such as the implantation rate, clinical pregnancy rate, and live birth rate) (Sauer *et al.*, 1990; Thomas *et al*., 2010; Capalbo *et al.*, 2017; La Marca *et al.*, 2017; Pirtea *et al*., 2020). Thus, the ova age was considered as a potential confounding factor in this study.

However, this study indicated that mean ova age in Group A and B were similar (30.60 vs. 30.27) and not statistically different. Furthermore, both average ages were well below the age of 38 years old associated with good-prognosis patients resulting from female age (Fleming *et al*., 2015, La Marca *et al.*, 2017).

4.2.4. Number and maturity of ova obtained from aspiration

Controlled ovarian hyperstimulation, utilized in ART cycles, aims to generate multiple mature oocytes (Law *et al*., 2019). The number and maturity of these ova are important confounding factors to consider when comparing the two groups' blastulation and ART outcomes (Committee of Gynaecologic Practice, 2015; Law *et al*., 2019). Where the number of ova (resulting from either poor or excessive ovarian response) is associated with a reduction in the fertilization rate, blastulation rate, proportion of good-quality blastocysts, day 5 embryo transfer rate, implantation rate, clinical pregnancy rate, and live birth rate (Sauer *et al.*, 1990; Lawler *et al.* 2007; Thomas *et al*., 2010; Liu and Case, 2011; Fleming *et al*., 2015; Capalbo *et al.*, 2017; La Marca *et al.*, 2017). A high proportion of immature oocytes is reported to reduce fertilization rates, blastulation rates, and good quality blastulation rates. Furthermore, it increases complex blastocyst mosaicisms correlated to poorer implantation potential, lower clinical pregnancy and live birth rates, and increased miscarriage rates (Lanzendorf *et al*.,1990; Strassburger *et al*., 2010; Shin *et al*., 2013). Thus, the average number and maturity of ova obtained were also considered potential confounding factors for this study.

In this study, the average number of ova obtained for Group A and B was 12.68 and 10.82, respectively (consisting of 10.82 and 11.53 mature ova, and 1.86 and 1.55 immature ova, respectively). This indicated that the cohort consisted of ova obtained from women with a good ovarian reserve, responded well to stimulation, and had a good prognosis of ART treatment based on the number and maturity of the ova obtained (Sunkara *et al.*, 2011; Committee of Gynaecologic

Practice, 2015; Law *et al*., 2019). Furthermore, there was a lack of statistical significance determined between the mean total, mature, and immature number of ova obtained for Group A and B.

4.2.5. Fertilization rate

The fertilization rate is another important potential confounding factor to consider when evaluating blastulation and ART outcomes between the two groups. In this study, the fertilization rate was defined as the percentage of mature ova observed to be normally fertilized 16-18 hours following IVF or ICSI insemination. This definition was adapted from the Vienna Consensus to incorporate both IVF and ICSI cycles, as both Group A and B retained similar ART utilization profiles, inferring insignificant differences, between the percentages of IVF and ICSI cycles employed (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

Nevertheless, the fertilization rate is known to be a possible confounding factor as differences in fertilization rates between two groups can be correlated to either difference in the cycle outcomes (such as gamete quality, number of zygotes obtained, blastulation outcomes, and ART outcomes) or laboratory standards (such as adherence of good laboratory practices and the standardization of reproductive scientists' technical skills to perform the chosen ART over the study period) (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). The fertilization rate was thus considered as an important potential confounding factor for this study.

However, the mean fertilization rate obtained for Group A and B was similar (82.25% vs. 80.83%) and were not statistically different. Furthermore, both percentages were above the benchmark value of ≥80% reported for normal ICSI fertilization rates in the Vienna consensus (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). This deduced that both groups were similar in both gamete fertilization potential and technical implementation of ART over the 3-year study period.

4.2.6. Day of embryo transfer

The day of embryo transfer refers to the number of days an embryo is cultured before transfer. Although there are reported implications that a longer *in vitro* incubation period (up until blastocyst stage) being associated with a higher percentage of preterm delivery, larger gestational age babies, an increased percentage of monozygotic twins, and an alteration in the sex ratio when compared a shorter *in vitro* incubation period (of cleavage stage embryos) (Maheshwari *et al.*, 2016). Several studies have reported a strong correlation between the duration of embryo culture and the

likelihood of ART success (with an equivalent number of embryos) (Gardner *et al.*, 2008; Peña *et al.*, 2018).

Increased ART success is attributed to the advantaged of prolonging the embryo culture (from the previously utilized 2-3 days of embryo culture to the now routinely accepted 5-7 days) (Peña *et al.*, 2018) resulting from the embryologist's ability to better select blastocysts with the greatest implantation potential and highest likelihood of obtaining beneficial ART outcomes from the cohort of available embryos (Gardner *et al.*, 2008; Peña *et al.*, 2018). Therefore, the day of embryo transfer is an important confounding factor to consider. However, as this study specified a fresh day 5 embryo transfer as one of the inclusion criteria, this factor was the same for both groups.

4.2.7. Number of embryos transferred

The number of embryos transferred refers to the number of embryos placed back into the recipient's uterus during embryo transfer. Various researchers have indicated that the number of embryos transferred directly affects the clinical pregnancy rate, miscarriage rate, and live birth rate (Thurin *et al.*, 2004; McLernon *et al*., 2010; Pirtea *et al*., 2020). At present, it is suggested that either one or two embryos (usually blastocysts) are transferred depending on both the patient prognosis and the developmental stage and quality of available embryos (Thurin *et al.*, 2004; McLernon *et al*., 2010; Pirtea *et al*., 2020). Thurin *et al.* (2004) supports the transfer of two embryos to significantly increase the clinical pregnancy rate whereas McLernon *et al*. (2010) recommends the use of elective single embryo transfer to increase the live birth rate by reducing both the miscarriage rate and risks associated with multi-fetal pregnancies.

In this study, the mean number of embryos transferred was similar for Group A and B (1.88 vs. 1.82) and not statistically different Furthermore, both values were within the recommended range of transferring one to two embryos (Thurin *et al.*, 2004; McLernon *et al*., 2010; Pirtea *et al*., 2020).

STRENGTHS AND LIMITATIONS OF STUDY

This study **fulfilled its aim** by successfully evaluating the effects of changing the embryo culture method at Drs. Aevitas Fertility Clinic. It met **the objectives** by comparing both the blastulation and ART outcomes for the two embryo culture methods utilized. Furthermore, with positive economic, practical, and risk-related advantages of co-culturing embryos in continuous media, coupled with the **statistically significance results obtained for the blastulation outcomes,** and the **positive trends obtained for the ART outcomes**, this study **supported the continued use of Embryo Culture Method B** for future ART cycles of Drs. Aevitas Fertility Clinic.

However, there are several limitations of this study that should be considered.

First, this was a **retrospective study**. As such, this study was designed to analyse pre-existing data, which by nature, is subject to bias and cannot be pre-defined and standardized (Sedgwick, 2014). The inability to design this study prevented us from comparing the effects of co-culturing the embryos and utilizing continuous culture media separately. Therefore, although the entire culture method could still be compared, it was unfortunate that the differences in blastulation and ART outcomes could not be associated with a specific modification or as a function of the combination thereof.

Second, the **sample size** is relatively small (479 cycles, consisting of 184 cycles in Group A and 295 cycles in Group B) (Section 2.2). Furthermore, it was hypothesized that the limited sample size might have resulted in the lack of statistical significance obtained for ART outcomes in this study (Section 4.2.2).

Third, **propensity score** matching was not performed. Although confounding factors were identified and assessed to ensure that the groups were balanced and comparable, it does not eliminate unforeseen bias in the population (Section 4.1; Austin, 2011). The use of propensity score matching could have been useful in reducing or eliminating unforeseen population bias between the two groups in this study (Austin, 2011).

Fourth, **cumulative ART outcomes** (such as cumulative pregnancy rates and live birth rates) were not assessed. Thus, although it was hypothesized that co-culturing embryos in continuous media were more likely to retain better cumulative ART outcomes (based on the blastocyst attribution profiles), these results could not be confirmed (Section 4.2.2).

Fifth, the **aneuploidy rates** were not considered. This factor could have however affected ART outcomes and be an alternative reason for the lack of significance obtained (Alfarawati *et al*., 2011; Zhao *et al*., 2018).

CONCLUSION OF STUDY

The aim of this study was to evaluate the effects of changing the embryo culture method, from individually culturing embryos in sequential media (Embryo Culture Method A) to co-culturing embryos in continuous media (Embryo Culture Method B) at Drs. Aevitas Fertility Clinic. This was performed by retrospectively assessing both the blastulation and ART outcomes over a three-year study period (January 2016-December 2018).

The results showed that Embryo Culture Method B retained significantly better blastulation outcomes (significantly better BDRs, a significantly better proportion of good-quality blastocysts, and a significantly higher day 5 embryo transfer rate). Furthermore, Group B retained a significantly better blastocyst attribution profile, resulting in significantly more and better good-quality blastocysts obtained, utilized, and available for cryopreservation. This suggested a potential advantage of attaining both better ART outcomes and cumulative ART outcomes.

However, following the fresh embryo transfers, although there was a positive trend in improved ART outcomes for Group B (higher implantation rates, higher clinical pregnancy rates, lower miscarriage rates, and higher live birth rates), these results were not significant. Thus, the ART outcomes extrapolated that, at worst, no detrimental results were attained following the change in the embryo culture system.

Together with the positive economic, practical, and risk-related advantages of co-culturing embryos in continuous media, these findings thus **support the continued use of Embryo Culture Method B in further ART cycles at Drs. Aevitas Fertility Clinic.** As not only will the utilization of this culture method benefit the running of the ART laboratory, but it is hypothesized to significantly improve the patients' probability of ultimately achieving a live birth from a single cohort of oocytes.

RECOMMENDATIONS FOR FUTURE STUDIES

There are several recommendations for future studies.

First, it would be advantageous to design and perform a **randomized prospective sibling study utilizing single embryo transfer**. In doing so, the researchers could design and analyse data based on well-defined and standardized data to ensure that it is reported clearly and consistently. Furthermore, the study could be designed to assess the two aspects of the embryo culture individually so that any significant differences in the blastulation or ART outcomes could be associated with either the change in culture media (from sequential to continuous media), the change in embryo culture method (from induvial culture to co-culture), or a combination thereof. In addition, the utilization of a sibling study would reduce the risk of population bias, whereas the use of single embryo transfer would maximize the chance of implantation for each patient with an embryo from either group while enabling the ART outcomes to be reliably traced back to either group.

Second, if it is decided to perform additional retrospective studies, it is worth considering the use of **propensity score matching** to reduce the unintentional population bias associated with retrospective studies.

Third, it would be preferable to increase the **sample size** to improve the chance of obtaining statistically significant ART outcomes. This could be easily performed by increasing the study period from three to five years (January 2015-December 2019) or including frozen embryo transfer cycles. This would be further beneficial as it would utilize freeze-all cycles (in which transfer is delayed) and enable the **cumulative ART outcomes** to be assessed. However, to do this, all blastocysts (blastulating on day 5 to 7) and not only those blastulating on day 5 should be considered.

Last, it may be worth investigating the **ploidy rate** of embryos for patients who underwent pre-genetic testing for aneuploidy (PGT-A) during the study period to determine whether the difference between the blastulation and ART outcomes may be associated with an increased aneuploidy rate in embryos co-cultured in continuous media.

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APPENDICES

Appendix A: Controlled ovarian hyperstimulation

Ovarian stimulation medication is monitored and adjusted by Drs. Aevitas Fertility Clinic's fertility specialists. Briefly, a standard antagonist stimulation protocol consists of daily gonadotropins (225 IU FSH in a step-down fashion to 150 IU) for five days, beginning on day 3 of the menstrual cycle. Adjustments to the gonadotropin dosage are determined by ultrasound monitoring. 0.25 mg of Cetrorelix® (GnRH antagonist) is added as a subcutaneous injection when the leading follicle measures 14 mm or more. 10,000 IU hCG is given subcutaneously or intramuscularly when the lead follicle was ≥18 mm, and at least two other follicles were ≥16 mm in size. Oocyte retrieval is performed within 36 hours after hCG administration.

Appendix B: Oocyte retrieval and pick-up

Oocytes are retrieved from female patients by Drs. Aevitas Fertility Clinic's fertility specialists utilizing aspiration. Here the follicular fluid is obtained using a long 16-gauge aspiration needle and a suction pump (100-120 mm Hg) into sterile tubes. After the aspiration of each follicle, the tube containing the follicular fluid is sent to the laboratory and given directly to an examiner or placed in a heated block at 37°C. Once a tube has been received, pour the follicular fluid into a large petri dish under the stereomicroscope fitted with a heated stage (37-40°C). Examine the follicular fluid immediately as red and white blood cells are prone to attach firmly to the cumulus cells. If the cumulus cells are excessively stained with blood, remove these areas with sterile needles as it is well established that the blood interferes with the fertilization rate and subsequent embryo quality. Identify cumulus-oocyte-complexes [COC] and grade each oocyte as metaphase I [MI] or metaphase II [MII]. Collect the COC, with as little blood and medium as possible, using a sterile, rounded, wide-bore glass pipette and transfer the COCs into the appropriate tubes (MI or MII, each containing 2.0 ml of Quinn's Advantage™ Medium with HEPES (SAGE™) pre-heated to 37°C). Wash and transfer the COCs into a Greiner dish containing 2.0 ml of Fert™ (ORIGIO®) (pre-incubated overnight at 37°C and 6% CO_2). Incubate in a PLANER CO_2 benchtop incubator (ORIGIO®) at 37°C and 6% CO_2, until time of denuding or insemination.

Appendix C: Semen preparation

Two methods of sperm preparation are employed at Drs. Aevitas fertility clinic, namely the wash and swim-up method and the discontinuous gradient centrifugation method. The method chosen is depended on the quality of the semen sample produced. Although the quality of the semen sample can be predicted by referring to a recent semen analysis, the quality of the semen is confirmed on the day of production via visual or microscopic analysis and adjusted as needed. Factors that may influence the method chosen include percentage motile sperm, rate of forward progression, concentration (total count), and the number of other cells in the semen sample. In addition, the assisted reproduction procedure being followed post semen preparation will play a role in the decision making. Whereas with in vitro fertilization [IVF], you may need 100 000 to $1x10^6$ spermatozoa per oocyte, you only need a single spermatozoon per oocyte for an intracytoplasmic sperm injection [ICSI] procedure.

The standard wash and swim-up procedure remains the most used procedure for sperm preparation as no foreign particles are introduced into the sample, the sample is free of other cells, and the percentage of motile sperm is high. The discontinuous gradient centrifugation method is preferred when dealing with samples where the spermatozoa are in low concentrations or have poor motility. Additionally, it is utilized to prepare samples with a high viscosity, high concentrations of other cells or debris contain a known viral contaminant, or where the sample has been cryopreserved.

The wash and swim-up procedure is most commonly used for all samples with a good concentration of spermatozoa ($\geq 15x10^6$), enough highly motile spermatozoa (≥30% with ≥2+ FP), and a low concentration of other cells (≤5 cells per field of view), as it does not introduce foreign particles into the sample; the sample is free of other cells, and the percentage of motile sperm is high. The discontinuous gradient centrifugation method was used for the more difficult samples which either did not meet the above criteria, have been cryopreserved or have known viral contaminants. This method can thus be used to isolate low concentrations of sperm with poor motility, yield sperm with less DNA damage due to eliminating reactive oxygen species [ROS] early on in the preparation method and remove cryoprotectants and viral contaminants.

1. Wash and swim-up method [ICSI/PICSI/IMSI/IVF]

Prepare three 15 ml round-bottomed tubes (labeled semen, SWM, and sperm) with the patent's surname and initials. Label all tubes and lids with a colour sticker to prevent preparation error. On

the sterile tube labeled sperm, add an additional label showing both partners' initials, surname and identification number, as well as the spermatozoa donor's code where applicable. Place 5 ml of Quinn's™ Sperm Washing Medium (SAGE™) [SWM] into the sterile tube labeled SWM and incubate all the tubes to 37°C before use. On completion of semen liquefaction in a sterile semen container, transfer the semen into the sterile tube labeled semen. Dilute the semen 1:2 with SWM and centrifuged at 350 to 400 xg for 10 minutes. Aspirate the supernatant and discard in the semen container. Resuspend the pellet in 2 ml of SWM and centrifuged at 350 to 400 xg for 10 minutes. Following centrifugation, aspirate the supernatant as close as possible to the pellet and discard in the semen container. Carefully overlay the pellet with 0.5 ml SWM while taking care not to disturb the pellet. Place the test tube at an approximate 45° angle at 37°C for 30-60 minutes. Note, for IVF procedures, the final 0.5 ml SWM for the swim-up step is replaced with Fert™ (ORIGIO®) (pre-incubated at 37°C and 6% CO_2 overnight). Once added, the tube should be placed back in the incubator to allow spermatozoa to swim-up for 30-60 minutes. After the swim-up period, carefully aspirate the top two-thirds of the SWM containing motile spermatozoa and transfer into the sterile final tube labeled sperm. Use the spermatozoa within 1 hour after the preparation has been completed. When faced with a problem sample, follow the above procedure can be modified as follows. The number of test tubes the sample is divided into can be increased with a subsequent decrease in the volume of medium overlaid on each pellet. Different test tube shapes can also be employed. In cases of very low initial concentrations, the standard flat-bottomed tubes can be replaced with conical tubes.

2. Discontinuous gradient centrifugation method [ICSI/PICSI/IMSI/IVF]

The discontinuous gradient employs the use of different concentrations of SilSelect (FertiPro). At the beginning of each week prepare 3 stock solutions and store at 4°C: 90% solution (9.0 ml of 100% SilSelect (FertiPro) and 1.0 ml of SWM), 70% solution (7.0 ml of 100% SilSelect (FertiPro) and 3.0 ml of SWM), and a 45% solution (45%: 4.5 ml of 100% SilSelect (FertiPro) and 5.5 ml of SWM). For each semen preparation prepare two 15 ml round-bottomed tubes (labeled semen and SWM) and two 15 ml conical tube (labeled gradient and sperms) with the patent's surname and initials. Label all tubes and lids with a colour sticker to prevent preparation error. On the sperm tube, add an additional label showing both partners initials, surname and identification number, as well as the spermatozoa donor's code where applicable. Place 5 ml of SWM into the sterile tube labeled SWM and incubate all the tubes to 37°C over night. Approximately 20 minutes before use, prepare the gradient in the sterile tube labeled gradient and incubate at 37°C. A two layered gradient of 90%

and 45% SilSelect stock solutions is used for all semen samples unless there is a known viral contaminant, or the sample was obtained from a testis biopsy. In these cases, the 70% layer is employed to ensure for greater separation of the sample. In general, I ml layers are used for IVF samples and 0.5 ml layer are used for ICSI/PICSI/IMISI. It should be noted that the gradient should be used within 2 hours of preparation, as additional time can result in the mixing of the gradient layers. On completion of semen liquefaction in a sterile semen container, transfer the semen into sterile tube labeled semen. Dilute the semen 1:2 with SWM and centrifuged at 350 to 400 xg for 10 minutes. Aspirate the supernatant and discard in the semen container. Resuspend the pellet in 0.5 ml SWM and carefully overlay onto the gradient. Note, in cases where the semen sample is less than or equal to the volume of each layer in the gradient, the semen can be overlaid onto the gradient directly. Centrifuge the gradient tube for 15 minutes at 300 xg. After centrifugation, carefully aspirate the top layer(s) and discard in the semen container, so that the pellet with ± 0.5 ml SWM remains. Transfer the pellet and medium into the sterile tube labeled sperm and wash the pellet twice with 2 ml SWM by centrifugation 400-450 xg for 10 minutes each. After completion, resuspend the pellet in 0.5 ml SWM. Use the spermatozoa within 1 hour after the preparation has been completed. When faced with a problem sample the volumes of the gradient can decreased or the number of gradients can be increased.

Appendix D: Oocyte insemination

Four methods of oocyte insemination employed at Drs. Aevitas Fertility Clinic from 2015-2019: in vitro fertilization [IVF], traditional intracytoplasmic sperm injection [ICSI], physiological intracytoplasmic sperm injection [PICSI] and intracytoplasmic morphologically selected sperm injection [IMSI]. The SOPs for these techniques are described below.

1. *In vitro* fertilization procedure

Make sure that all forms and documentation are prepared. Check the patient's file and records to eliminate all possible uncertainties/queries. After aspiration, transfer COCs to a Nunc™ 4-well dish (Thermo Fisher Scientific) containing Fert™ (ORIGIO®) (pre-incubated overnight at 37°C and 6% CO_2). Separate the oocytes by levels of maturity and ensure that not more than 5 complexes are placed in each well. Incubate in a PLANER CO_2 benchtop incubator (ORIGIO®) at 37°C and 6% CO_2 until insemination. Complete all forms. Once semen preparation has been completed (Appendix C), inseminate the complexes with the correct number/volume of prepared sperm. Where possible, insemination should be performed at ± 40 hours post hCG administration. Spermatozoa with a morphology of ≤4% should be added at a concentration of ≤2x10^6 sperm/ovum. Spermatozoa with a morphology of 4-14% should be added at a concentration of 500 000 sperm/ovum and spermatozoa with a morphology of >14% should be added at a concentration of 100 000 sperm/ovum. Incubate in a PLANER CO_2 benchtop incubator (ORIGIO®) at 37°C and 6% CO_2.

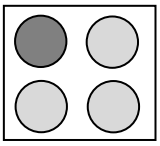
2. Intracytoplasmic Sperm injection procedures

Three types of intracytoplasmic sperm injection techniques are used at Drs. Aevitas Fertility Clinic: traditional ICSI, PICSI and IMSI. For all these techniques make sure that all forms and documentation are prepared. Check the patient's file and records to eliminate all possible uncertainties/queries.

Denuding oocytes

Denuding should be performed at ± 38 hours post hCG injection when possible. To denude the oocytes, prepare the following pipettes: a fire polished glass Pasteur pipette, a hand drawn glass pipettes and the Cook stripper (Marcus Medical) pipette. Then prepare one Nunc™ 4-well dish (Thermo Fisher Scientific) dish for every 8 oocytes (as depicted below). This is done by placing 0.3 ml SAGE™ IVF Inc. Hyaluronidase (80 UI/ml) and 0.6 ml Quinn's Advantage™ Medium with HEPES (SAGE™) into well 1, and ± 0.7 – 0.8 ml of Quinn's Advantage™ Medium with HEPES (SAGE™) into

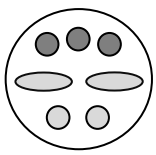
wells 2, 3, and 4. Place the dish at 37°C (without CO_2) for at least 10 minutes. After warming, place up to 8 oocytes into well 1 for ± 30 seconds. Gently flush the complexes with a standard fire polished pipette until all cumulus cells are digested (oocytes with corona cells and small number of cumulus cells form "fluffy balls"). If complexes stay intact use two hypodermic needles to "tease" oocytes from the complexes. Use the same fire polished pipette and transfer the oocytes with as little medium from well 1 into well 2. Now flush the oocytes individually with a pulled glass pipette (170-200 µm) to remove some of the loose cumulus cells and transfer to well 3. In well 3, start the denuding the oocytes using the plastic Cook denuding pipette (130 µm) (Marcus Medical). Try to remove all corona cells to allow for evaluation of oocyte maturity. Make sure that the pipette works correctly before oocytes are aspirated, to prevent oocytes from getting stuck in the pipette. If oocytes seem stuck, blow out into one of the wells using a syringe and adapter.



Denuding dish

- Hyaluronidase and Quinn's Advantage™ Medium with HEPES mixture
- Quinn's Advantage™ Medium with HEPES

After denuding, place the oocytes into the left most elliptical Fert™ (ORIGIO®) drop of a holding/rugby ball dish. The holding/rugby ball dish should be prepared with Fert™ (ORIGIO®) and/or Cleav™ (ORIGIO®) (as depicted below) and covered in Oil for Tissue Culture (SAGE™). The top three culture drops are made from Cleav™ (ORIGIO®) when following the sequential culture media protocol (Group A). Whereas the top three culture drops are made from Fert™ (ORIGIO®) when following the 1-Step continuous culture medium protocol (Group B) (Appendix E). Note: Pre-incubate the dish in a PLANER CO_2 benchtop incubator (ORIGIO®) at 37°C and 6% CO_2 before use. After washing, transfer the oocytes to the right most elliptical Fert™ (ORIGIO®) drop, and determine the oocytes' maturity. All MII oocytes should be placed in the top left medium drop, all MI should be placed in the lower left medium drop, and all the GVs should be placed in the lower right medium drop. Place the dish back in the incubator until insemination is performed.



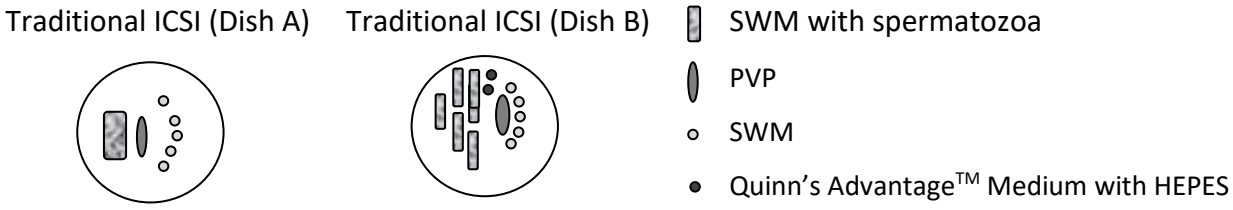
Holding/rugby ball dish

- Cleav™ (Group A) /Fert™ (Group B)
- Fert™
- Fert™

Preparation of specific ICSI dishes for traditional ICSI, PICSI and IMSI

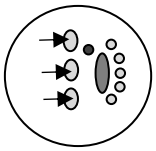
Make an ICSI dish for the specific type of ICSI procedure performed. Switch on the heated stage, set-up the holding and injection pipettes, and calibrate the manipulators.

***Traditional ICSI*:** Prepare 1 of 2 dishes using Quinn's™ Sperm Washing Medium (SAGE™) [SWM], SAGE™ IVF Inc. Polyvinylpyrrolidone (7% solution) [PVP] and Quinn's Advantage™ Medium with HEPES (SAGE™), depending on the sperm sample results post-preparation (Appendix C). For standard sperm samples, prepare Dish A. For testicular/severe oligozoospermia samples, prepare Dish B. Cover drops completely with Oil for Tissue Culture (SAGE™) and store the dish at 37 °C for at least 30 minutes before use.



PICSI: Prepare the PICSI® dish with Hyaluronan microdots (ORIGIO®) (Dish C). Take special care when hydrating the hyaluronan microdots. Briefly, hydrate the hyaluronan microdots by placing single 10 µl elliptical droplets of sperm preparation medium (SAGE™) at the end of each locating line covering the area where the microdot is situated. Then add the PVP and Quinn's Advantage™ Medium with HEPES (SAGE™) drops and carefully flood the dish with Oil for Tissue Culture (SAGE™). Leave for ± 5 minutes and add a small volume (10 µl) of prepared sperm to the first hyaluronan microdots by touching the tip of the micropipette containing the sperm to the edge of the hydrating drop at the bottom of the dish under the oil and expel the sperm. By delivering the sperm in a volume equal to the hydrating fluid, immediate mixing and delivery of sperm to the vicinity of the microdot is assured. If the sperm are delivered in a smaller volume at the edge of the drop, greater than 30 minutes may be required for them to swim through the hydrating fluid to the microdot. Alternatively, the sperm suspension can be added directly to the dry microdot. Sperm binding begin normally in 5 minutes or less. Some microdots may require 30 minutes or more to reach full binding capability Therefore, whenever marginal sperm binding is observed, pre-hydrate for 30 minutes or more, or allow sperm to incubate on the dot for 30 minutes or more before selecting sperm.

ICSI (Dish 3): PICSI dish



- Hyaluronan drops with spermatozoa
- SWM
- PVP
- Quinn's Advantage™ Medium with HEPES

Special considerations:

1. Microdot shape: The PICSI® Sperm Selection Device hyaluronan microdot is crater shaped. The edge of the microdot is a raised wall of hydrogel surrounding a low, flat interior layer. The wall is flexible and may be irregular in shape due to uneven hydration of the hydrogel. The hydrogel wall can be pierced and torn by an ICSI micropipette driven directly into it. It is best to position the elevated micropipette tip over the microdot interior and lower it to the microdot surface for recovery of sperm.

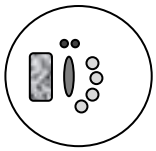
2. Microdot caves: During manufacturing, uneven hydration may cause segments of the microdot wall to create small "caves" that open toward the inside edge of the wall. Sperm that swim into a cave are trapped, not bound. Trapped sperm usually all face away from the center of the microdot and show vigorously beating tails, often in clusters. The heads of trapped sperm can move laterally and sometimes back and forth within the walls of the cave. Trapped sperm should not be selected since their binding status is unclear.

3. Microdot stability: If a part of the wall separates from the polystyrene, the same forces that create caves can cause the microdot wall to progressively detach from the dish and coil up like a spring. When this occurs, some or all the wall will separate from the microdot. However, the microdot interior hyaluronan layer will remain intact. The interior hyaluronan layer is stable for hours, it collects, and houses bound sperm that may be used for ICSI. Sperm bound to the curled-up wall remnant should not be used for sperm selection and isolation.

4. Temperature: Sperm bind best to hyaluronan hydrogel at temperatures below 30°C. At temperatures above 30°C, sperm swimming vigor increases, and the swimming force may overcome the binding force. The result is that about one-third of sperm bound at room temperature will show some progressive migration at 37°C and may be deemed not bound and therefore immature. PICSI® Sperm Selection Device dishes placed on a 37°C heated stage will come to about 33°C and then remain at that temperature. Therefore, select bound sperm at room temperature, store in sperm prep drop and warm to 37°C before final injection.

***IMSI*:** Prepare Dish D. After drop preparation, cover completely with Oil for Tissue Culture (SAGE™) and store the dish at 37°C for 30 minutes before use.

IMSI (Dish D)



SWM with spermatozoa

SWM

PVP

Quinn's Advantage™ Medium with HEPES

Sperm Selection and Immobilization for traditional ICSI, PICSI and IMSI

Spermatozoa are selected depending on the type of ICSI used, as indicated below.

***Traditional ICSI*:** For standard spermatozoa samples, select enough motile, morphological normal spermatozoa for the injection procedure with the ICSI injection pipette and immobilize the sperm cell. For testicular spermatozoa, select moving or twitching sperm for the injection procedure with a testis biopsy pipette with an inner diameter of ± 15 µm. Remember to fill this pipette with PVP before aspirating the sperm to prevent the sperm from getting stuck. Once obtained, place the sperm in the small prep drop and repeat until enough sperm has been selected (ideally 2x more that the number of oocytes to be injected). Reheat to 37°C before injecting.

***PICSI*:** Select Hyaluronan bound sperm. These sperm are easily to identify by exhibiting circular motion around the drops, caused by vigorous tail beating. It is sometimes difficult to distinguish whether the sperm are bound or simply swimming against the edge of the microdot. Therefore, select sperm from the interior of the microdot. If the density of bound sperm is too high or too low for good sperm selection, dilute or concentrate the prepared sperm sample and use the adjusted sperm sample to seed the next microdot. Three microdots are provided on each PICSI® Sperm Selection Device to give enough opportunity to obtain the correct dilution and obtain the required number of spermatozoa. To collect a bound sperm, position the tip of the ICSI micropipette next to the sperm and gently suck fluid into the pipette, drawing in the sperm. Place in the small reservoir drop of sperm prep. Continue collecting until 20-50 sperm are captured. Aspirate a single selected sperm cell and place in the PVP and immobilize.

***IMSI*:** Select motile, morphological normal spermatozoa from the drop with the ICSI injection pipette using the 20x objective. Place the selected sperm into the left sperm preparation drop and focus on the edge of the drop. Change the heated stage to the metal one with a hole, to ensure that the dish meets the objective. Put the 100x objective in place and add a small drop of oil onto the objective.

Place the glass bottom dish containing the sperm onto the oil-covered objective. The left sperm preparation drop should be in the center of the objective. Use the 100x magnification to focus on the sperm preparation drop edge. Bring the needle down and make an indentation in the drop edge. The sperm will swim into the indentation. Select morphologically normal sperm without any vacuoles and move them to the sperm preparation drop to the right.

Spermatozoa injection

Place 2-3 MII oocytes to be injected into Quinn's Advantage™ Medium with HEPES (SAGE™) drop. Select an immobilized sperm cell and carefully inject oocyte. Repeat until all oocytes have been injected. Then, transfer the oocytes back into the left elliptical drop in the holding dish to wash and then into the top drops for overnight culture at 37°C and 6% CO_2. Where possible, inject the oocytes ± 40 hours post hCG administration.

Appendix E: Embryo culture and evaluation

This study investigates fresh ART cycles performed at Drs. Aevitas Fertility Clinic from 2015-2019. It should thus be noted that the clinic's SOP about embryo culture was modified in 2017. Embryos cultured until the change in the protocol were individually cultured in the Sequential Series™ embryo culture medium (ORIGIO®) using the sequential media protocol. Whereas embryos cultured after the change were co-cultured in SAGE™ 1-Step™ with Human Albumin Solution (ORIGIO®) using the continuous culture medium protocol.

1. Sequential Media Protocol

Post-insemination, oocytes were cultured overnight in Cleav™ (ORIGIO®) as described in Appendix D.

On day 1, check fertilization. In the case of ICSI patients, oocytes can be checked as is. In the case of IVF patients, clean the oocytes using denuding pipettes and rinse well before determining fertilization. Visualize the presence of pronuclei [PN] and polar bodies [PB] on an inverted microscope fitted with heated stage. (Take note whether PN numbers are abnormal at > or <2, or any other anomalies). Fertilization is indicated by the presence of 2PN. Record fertilization and transfer the embryos from the holding/rugby ball dish to individual Quinn's Advantage Protein Plus™ Cleavage Medium drops covered with SAGE® Oil for Tissue Culture in a Greiner dish and incubate at 37°C and 5% CO_2 until day 3.

On day 3, check embryo development, by determining the number cells within the embryo. Embryos are expected to be at the 6- to 8-cell stage. Record the number of cells and any anomalies. Transfer the embryos into Quinn's Advantage Protein Plus™ Blastocyst Medium drops covered with SAGE® Oil for Tissue Culture in a Greiner dish and incubate at 37°C and 5% CO_2 until day 5.

On day 5, check the development of the embryos as described in Appendix F. The best quality blastocyst(s) are selected for fresh embryo transfer (Appendix G). Cryopreserve the remaining good quality blastocysts for potential use in future frozen embryo transfers as per the patient's consent. Transfer any developing embryos, which have not yet reached blastocyst stage, into fresh Quinn's Advantage Protein Plus™ Blastocyst Medium drops covered with Oil for Tissue Culture (SAGE™) (in a Greiner dish). Incubate these embryos in a PLANER CO_2 benchtop incubator (ORIGIO®) at 37°C and 6% CO_2 for another 2 days.

On day 6 and 7, check the embryos for blastulation and either transfer or cryopreserve the blastocysts as described above. Discard any embryos which have not become blastocysts by day 7.

2. Continuous Culture Medium Protocol

Post-insemination, oocytes were cultured overnight in Fert^{TM} (ORIGIO®) as described in Appendix D.

On day 1, check fertilization. In the case of ICSI patients, oocytes can be checked as is. In the case of IVF patients, clean the oocytes using denuding pipettes and rinsed well before checking for fertilization. Visualize the presence of pronuclei [PN] and polar bodies [PB] on an inverted microscope fitted with heated stage. (Take note whether PN numbers are abnormal at > or <2, or any other anomalies). Fertilization is indicated by the presence of 2PN. Record fertilization and transfer the embryos from the holding/rugby ball dish to SAGE^{TM} 1-Step^{TM} with Human Albumen Solution media (ORIGIO®) drops (≤5 embryos/drop) covered with SAGE® Oil for Tissue Culture in a Greiner dish and incubate at 37°C and 5% CO_2 until day 5.

On day 5, check the development of the embryos as described in Appendix F. The best quality blastocyst(s) are selected for fresh embryo transfer (Appendix G). Cryopreserve the remaining good quality blastocysts for potential use in future frozen embryo transfers as per the patient's consent. Transfer any developing embryos, which have not yet reached blastocyst stage, into fresh SAGE^{TM} 1-Step^{TM} with Human Albumen Solution media (ORIGIO®) drops (≤5 embryos/drop) covered with Oil for Tissue Culture (SAGE^{TM}) (in a Greiner dish). Incubate these embryos in a PLANER CO_2 benchtop incubator (ORIGIO®) at 37°C and 6% CO2 for another 2 days.

On day 6 and 7, check the embryos for blastulation and either transfer or cryopreserve the blastocysts as described above. Discard any embryos which have not become blastocysts by day 7.

Appendix F: Blastocyst grading

Morphological grading

Morphologically grade all blastocysts on day 5 of embryo culture as close to 116 ± 2 hours post-insemination as possible. Each blastocyst should be graded using the following system.

For exceeding early blastocysts, where the inner cell mass [ICM] and trophectoderm [TE] cannot be distinguished clearly enough to be graded, the blastocysts should be designated one of the two following codes:

EB - denotes a blastocyst which has started cavitation but has not yet formed a blastocoel.

1B – denotes a blastocyst where the blastocoel can be visualized but has not yet filled more than half the volume of the conceptus.

For blastocysts, in which the ICM and TE can be distinguished clearly, the blastocysts should be designated a digit code representing the degree of expansion and hatching status, the quality of the inner cell mass (ICM), and the quality of the trophectoderm (TE), respectively.

Use the coding system below, modified from Schoolcraft *et al*. (1999):

Degree of Expansion and hatching status

1 - Early blastocyst, the blastocoel filled more than half the volume of the conceptus. However, there is no expansion in overall size compared to the early cleavage stage embryos.

2 - Blastocyst, the blastocoel filled more than half of the volume of the conceptus, with slight expansion in overall size and notable thinning of the zona pellucida.

3 - Full blastocyst, a blastocoel more than 50% of the conceptus volume, and overall size fully enlarged with a very thin zona pellucida.

4 - Hatching blastocyst, that did not undergo preimplantation genetic screening. The trophectoderm has started to herniate.

5 - Fully hatched blastocyst that did not undergo preimplantation genetic screening. Free blastocyst fully removed from the zona pellucida.

ICM grading

A - Tightly packed, compacted cells

B - Larger loose cells

C - No ICM, distinguishable

D - Cells of ICM appear degenerated

TE grading

A - Many healthy cells forming a cohesive epithelium

B - Few, but healthy cells, large in size

C - Poor, very large or unevenly distributed cells, may appear as few cells squeezed to the side

D - Cells of the trophectoderm appear degenerated

Blastocyst quality categorization

Assign all blastocysts to a quality category based on their morphological grading scores and the likelihood of implanting and going on to achieve a clinical pregnancy and live birth, as shown below.

Good blastocysts - Fully expanded blastocysts, larger in size than a cleavage-stage embryo with a thinning zona pellucida, a well-sized inner cell mass, and a cohesive trophectoderm.

Examples: 3-5AA, 3-5AB, 3-5BA, 3-5 BB.

Fair blastocysts - Fully formed blastocysts with a well-sized inner cell mass and a cohesive trophectoderm but have not yet expanded. The zona pellucida has thus not stating thinning.

Examples: 1-2AA, 1-2AB, 1-2BA, 1-2BB

Poor blastocysts – The remaining blastocysts. These include blastocysts that show evidence of cavitating, but the two distinct cell types are not yet visible; blastocysts where the blastocoel can be visualized but has not yet filled more than half the volume of the conceptus; and blastocysts with a small/no inner cell mass or an irregular/non-continuous trophectoderm and/or degenerated cells.

Examples: EB, IB, and all blastocysts containing a C or D grade for either/both the ICM and TE.

It should be noted that this method of characterization is modified from Richardson *et al*. (2015). However, the modified method, separates all the blastocysts into three (as opposed to five) groups. In doing so, early blastulating embryos (EB, 1B) have been grouped into the poor blastocyst category,

whereas compacted embryos (morulae, which are not considered as blastocysts) have been omitted from these analyses.

Appendix G: Embryo transfer

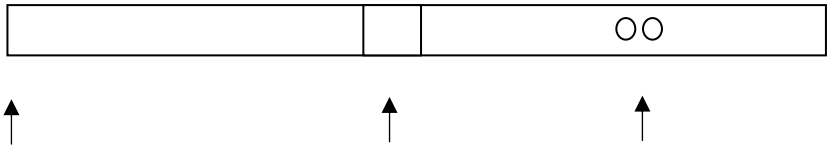
Preparation

Prepare the transfer dish. This consists of a Nunc™ 4-well dish (Thermo Fisher Scientific) with 0.8 ml blastocyst culture media in wells 2 and 3 and 2ml of blastocyst culture media in the middle to minimize evaporation in wells. The blastocyst culture media used depends on the embryo culture protocol. For embryos cultured using the sequential culture protocol, use ORIGIO® Sequential Blast™ (Group A). For embryos cultured using the 1-step continuous culture protocol use SAGE™ 1-Step™ with Human Albumen Solution media (ORIGIO®) (Group B). Incubate the dish overnight in a PLANER CO_2 benchtop incubator (ORIGIO®) at 37°C and 6% CO_2 before use.

Decide which and how many embryos will be transferred after consultation with the patients and the clinician. Place sterile instruments (forceps, speculum, valsellum), sterile gauze, and the transfer catheter and stylet on a sterile green cloth. Remember to place the stylet into the cannula of the soft catheter, so that it is ready for the clinician to use. Keep 5 ml Quinn's Advantage™ Medium with HEPES (SAGE™) medium incubated at 37°C ready for rinsing the speculum. Discuss the procedure and what to expect with the couple. Pictures of similar types of embryos can be shown to them. The patient will then be positioned on the bed so that she is comfortable, and the clinician has good access and vision to the vagina and cervix. The procedure starts by placing a speculum in the vagina to visualize the cervix, which is rinsed with sterile Quinn's Advantage™ Medium with HEPES (SAGE™) medium. The cervix is then cleaned to remove an old blood and mucus. The uterus is then visualized using an abdominal ultrasound and the embryo catheter loading and transfer can begin. Note: It is recommended that the patient's bladder is full before the transfer occurs as this ensures that the endometrial cavity can be accessed and visualized easily.

Embryo catheter loading

Once the clinician is ready for the transfer, with good sonar vision of the uterus cavity and confirmation that the cannula can be visualized in the correct place, load the embryo/s into a classic embryo transfer catheter (Wallace ®) as depicted below. Aspirate blastocyst medium from well 2 into a nontoxic 1ml IVF and artificial insemination [AI] syringe (Laboratorie CCD). Connect the soft catheter and expel the whole volume back into well 2. Make an air space of about 1 cm at the tip of the catheter. Visualize the embryo(s). Aspirate ± 10 µl of medium (± 4 cm) into the catheter and then the embryos until a total of ± 20 µl in total has been aspirated. Take to the clinician.



Tip of catheter air 20µl of medium with embryo(s)

Transfer procedure

A Wallace® Classic embryo transfer catheter (Cooper Surgical®), which contains the embryos to be transferred, is inserted through the cannula into uterine cavity. After insertion of the catheter, deposit the media containing the embryos into the uterine cavity. It is important that the embryos are placed in the correct position and that care is taken not to touch the fundus. After the deposit of the embryos, immediately check the catheter to ensure that the embryos did not remain inside the catheter. If the embryos are inside the catheter, transfer the embryos again as described above. Note: The transfer is guided with an abdominal ultrasound to ensure correct placement in the uterine cavity. Anesthesia is not required when performing an embryo transfer. It is recommended that the patient remains lying down for 15 minutes before she gets up to empty her bladder, avoids strenuous exercise, alcohol, caffeine, medication, cigarette smoke and sexual intercourse.

Appendix H: Consent forms

Consent to a medical procedure or examination

AEVITAS
Fertility Clinic

CONSENT TO MEDICAL PROCEDURE / EXAMINATION

NAME OF DOCTOR	Print Name _____ Signature Date	I have explained the nature, risks and possible consequences of the medical procedure to the undersigned patient or person legally competent to give consent.
MEANS USED TO EXPLAIN THE PROCEDURE	*Personally* *Via interpreter*	CIRCLE whichever is applicable.
NATURE OF PROCEDURE		
ANAESTHETIC	*Local* *Deep sedation*	CIRCLE whichever is applicable.
CONSENT TO USE OF BLOOD AND/OR BLOOD PRODUCTS	*Granted* *Withheld*	Granting or withholding of consent by the undersigned patient to the use of blood and/or blood products should it become necessary during the procedure. CIRCLE whichever is applicable.
I agree that a sample of my blood will be taken and tested for Hepatitis B and the Human Immunodeficiency Virus should an incident of contamination of a health care worker by bodily fluids occur during the procedure.		
FULL NAME OF PATIENT _____ Signature/Thumb-print of patient _____ Date _____		I, the undersigned, hereby consent to the performance of, and understand the nature, risks and possible consequences of the above procedure. The doctors who perform the above may increase the reasonable scope thereof or carry out additional or alternative measure (including general anaesthesia) if considered necessary.
PERSON LEGALLY COMPETENT TO GIVE CONSENT	Print Name _____ Signature _____ Date _____ Capacity or relationship to patient _____ Means by which consent was given: *Personally* *Telephonically* *Telegraphically*	This section to be filled in if consent is given by a person other than the patient.
WITNESS 1 WITNESS 2	Print Name _____ Signature _____ Print Name _____ Signature _____ Date _____	Names and signatures of witnesses to the signing of this document by the patient or a person legally competent to give consent on behalf of the patient.

Letter of consent by the donor

LETTER OF CONSENT BY THE DONOR

I hereby declare that the above given information is correct and that I will inform the unit of any change(s) in the above mentioned questions/statements. I also give my consent to the following:

1. a medical examination, which shall include a physical examination, by a competent person, if necessary;
2. questioning by a competent person related to my ova donation;
3. the collection of blood samples when necessary;
4. the removal of and use of my ova in the donor ova programme;
5. the testing and analysing of my ova and/or any other processing deemed necessary by a competent person;
6. the registration of my identification number with the Directorate of Health Services and the Central Data Bank;
7. my particulars, including my date of birth, age, height, mass, eye colour, hair colour, complexion, population group, nationality, sex, religion, occupation, highest educational qualification, and fields of interest, but excluding my identity, being made available to the parents/recipient and the Central Data Bank; and
8. providing details of my family history, especially with regard to any possible genetic condition or carrier status and mental illness in respect of any member of my family.

Note:
Blood samples collected will be used to identify any blood related diseases, i.e. HIV, Hepatitis B sAg & C Ab, RPR & TPHA, Chlamydia Trachomatis and Blood Group, AMH & Cystic Fibrosis.

I confirm that I have not given birth to more than 6 (six) children through artificial fertilisation using my ovum. Once I have had six live births, as contemplated herein, I consent to the further use of my ovum to achieve pregnancy by parents who have previously used my ova to achieve pregnancy and wish to have further children with the use of my ova. I understand that this will only be possible with the consent of the Minister. I further consent to my ovum being destroyed should six live births have occurred as the result of the use of my ovum.

I understand that the parents will be the owners of any embryos that are created with the use of my ova. I confirm that I am aware that they may donate their embryos to other recipients or for other purposes (other than embryo transfer). I confirm that a competent person may destroy embryos, created with the use of my ova if a parent/recipient has not claimed the embryo for 10 (ten) years.

The intention of donating ova is to achieve pregnancies.

I confirm that I have/ have not (please select) previously donated my ova.

I confirm that I donated my ova at _____(place) on the _____(date).

Signature(Donor): _____

Signature(Spouse): _____

Signature(Witness): _____

Signature(Witness):_____

Date: _____

Consent to treatment involving egg donation and embryo replacement as an egg recipient.

AEVITAS
Clinic

Dr Victor Hulme
MBChB, M.Med
(O+G) (Stell)

Prof Thinus Kruger
MBChB, MPharMed, MMed (O+G)
FCOG (SA), FRCOG (London), MD

Prof Igno Siebert
MBChB, MMed (O+G)
LKOG / FCOG (SA)

FertiliteitsSpesialiste&Ginekoloë / Fertility Specialists &Gynaecologists
PR No 0626546 / 7700539 VAT 4190266280

CONSENT TO TREATMENT INVOLVING EGG DONATION AND EMBRYO REPLACEMENT AS AN EGG RECIPIENT

.....
(Full name of husband / partner 1)

AND

.....
(Full name of wife / partner 2)

OF THE FOLLOWING ADDRESS:

.....

.....

.....

We, being husband/partner and wife/female partner of the above address hereby state:

1. We authorise **Prof. T.F. Kruger / Prof. T.I. Siebert / Dr. V.A. Hulme** (here after referred to as "the doctor") or suitable trained member of AEVITAS Clinic's staff to inseminate donor oocytes and transfer the obtained embryos to endeavour to cause the wife to become pregnant.

2. We authorise the doctor to obtain oocytes donated by:

   - an anonymous egg donor. Code.....Agency.....
   - a known egg donor. Name.....

Vincent Pallotti Hospital
Park Road, Pinelands, 7405
Tel: 021 531 6999 • Fax: 021 531 7919
PO Box 112, Howard Place, 7405 • Posbus 112, Howard Place, 7405

Email: medici@aevitas.co.za

3. The sperm to be used to endeavour to make the wife/female partner/surrogate pregnant will be that of:
   - the husband/male partner
   - an anonymous sperm donor. Code……..
   - a known sperm donor. Name……

4. The doctor has explained to us the nature and implications of the procedure and we understand that, even though the oocyte donation may be repeated as often as recommended by the doctor, there is no guarantee on his part that a pregnancy or full term pregnancy will result.

5. We understand that should we be married at the time of artificial fertilisation of the wife, we will be considered the parent/s of the child/children and will have full parental rights and responsibilities in respect of the child/children. Both the husband and the wife agree to maintain the child/children born as a result of the embryo transfer as if it was their own. Should we be unmarried at the time of artificial fertilisation of the female partner (recipient), the recipient will be considered the parent of the child/children.

6. We agree to rely upon the discretion of the doctor as to the suitability of the donor and we agree that we shall not be entitled at any stage to be informed as to the identity of an anonymous donor.

7. Fertilisation of fresh or frozen thawed eggs: We understand that the eggs in a fresh cycle will be fertilised immediately after retrieval. However we understand that eggs may be frozen after retrieval for fertilisation as a later specified date.

8. Cycle cancellation. We understand that failed fertilisation of eggs and failure to culture embryos is possible complication of IVF (*In Vitro Fertilisation*) treatment. We understand that there is also a rare risk of incubator failure, laboratory technical problems or problems with culture medium, which can affect our embryo culture. We understand that there is no guarantee that eggs will be retrieved. We accept these risks of IVF treatment and will not hold Aevitas Clinic or any of its staff responsible for these complications. We understand that we would not be responsible for all the costs of the treatment in these circumstances although we will be liable to pay the costs up to the point of cancellation of the cycle.

Vincent Pallotti Hospital
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Email: medici@aevitas.co.za

9. We also understand that there might be problems with the egg donor not adhering strictly to instructions resulting in cycle cancellation or the harvest of fewer eggs than expected. We accept that Aevitas Clinic cannot be held responsible for any problems due to donor non-compliance.

10. We understand that more than one embryo may be transferred, and this may increase the risk of a multiple pregnancy. We understand that multiple pregnancies have an increased risk of miscarriage, premature labour and an increased financial and emotional cost. We also understand that it is possible for an embryo to split leading to a multiple pregnancy.

11. We acknowledge that no more than two zygotes or embryos may be transferred to the wife during an embryo transfer procedure, unless there is a specific medical indication requiring the contrary.

12. We understand that if pregnancy results there is a possibility, as with any pregnancy, of complications of childbirth and delivery, or the birth of an abnormal child, or undesirable hereditary tendencies of such a child, or other adverse consequences, and we hereby waive any legal action which we may have against the doctor or any member of his staff or the donor in respect of such adverse results that may in any way have been caused by oocyte donation. Although the vast majority of children conceived with ART (Artificial Reproductive Therapy) are healthy, pregnancy after IVF is altered as evidenced by risk of preterm delivery, low birth weight among infants, and an altered prevalence of preeclampsia. In men with very low sperm count, utilizing ICSI (*Intra Cytoplasmic Sperm Injection*) treatment, there may be an increased prevalence of low sperm count in male offspring.

13. Any dispute or claim arising out of or in relation to this agreement or any breach thereof or any alleged derelict relating to the donor, shall be referred to confidential arbitration for decision by a single arbitrator to be agreed upon between the parties within seven (7) days of a dispute having been declared in writing. Should no such agreement be reached as to who shall act as an arbitrator, an advocate of no less than 10 (ten) years practical experience shall be selected to act as an arbitrator. The arbitrator shall act informally and shall not necessarily be bound by the provisions of the Arbitration Act No. 42 of 1965. Arbitration shall be held in Cape Town and finalized within thirty (30) days after his / her appointment.

14. The liability of the doctor for any and all claims in term of this Agreement shall be limited to a maximum amount of 2.5 million South African rand (ZAR).

Vincent Pallotti Hospital
Park Road, Pinelands, 7405
Tel: 021 531 6999 • Fax: 021 531 7919
PO Box 112, Howard Place, 7405 • Posbus 112, Howard Place, 7405

Email: medici@aevitas.co.za

15. We will have the benefit of a maximum of 15 eggs from our donor. Any eggs retrieved in addition to that number may be vitrified (frozen) and stored in an egg bank. We understand that if no eggs are retrieved from our chosen donor, we may have the choice of receiving frozen eggs from the egg bank, if available, at no additional charge.

16. We will not engage in any financial arrangements with the egg donor except directly through Aevitas Clinic.

17. We understand that we need to complete the necessary AEVITAS Clinic forms should we wish to:
    17.1 have our embryo/s stored for a further period for the purpose of subsequent embryo transfer to the wife/recipient; and/of
    17.2 have our embryo/s for transfer to another specific recipient; and/or
    17.3 have our embryo/s for a purpose, other than embryo transfer, which purpose must be stated at the time that our consent is given.

18. We agree to store our vitrified (frozen) embryos according to the protocols of Aevitas Clinic and acknowledge that Aevitas Clinic discard any embryos that are unclaimed for a period of **five (5) years**.

19. I, the wife, consent to:
    20.1 a physical examination and questioning by the doctor; and
    20.2 my particulars being made available to the Central Data Bank.

20. We confirm that we have / have not (please select) previously undergone artificial fertilisation and embryo transfer at _____(place) on the _____(date).

21. I, the wife, confirm that I will notify the doctor, in writing and within **30 (thirty)** days, should I give birth as a result of the artificial fertilisation. Furthermore I confirm that I will immediately notify AEVITAS Clinic should I become aware that my child may suffer from any mental illness or disorder.

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SIGNED AT.....ON .....DAY OF.....20 .....

1. HUSBAND / PARTNER: .....

2. WIFE / PARTNER: .....

3. WITNESS: (1).....

(2) .....

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Appendix I: Disclosure of use of clinic's name, and institutional approval for use of patient and medical records

DISCLOSURE OF USE OF CLINIC'S NAME, PATIENT/MEDICAL RECORDS & INSTITUTIONAL APPROVAL

To whom it may concern

We the partners of Drs. Aevitas Fertility Clinic, Life Vincent Pallotti Hospital, Alexander Road, Cape Town, hereby give consent that Dr. Taryn McLachlan (HPCSA registration number: MSIN0004723) can use de-identified routine medical/laboratory only records of patients treated at the clinic for her research study titled:

THE EFFECTS OF CO-CULTURING HUMAN EMBRYOS IN ONE-STEP CONTINUOUS CULTURE MEDIA ON ASSISTED REPRODUCTIVE TECHNOLOGY [ART] OUTCOMES

This consent further gives institutional approval by Drs. Aevitas Fertility Clinic for this study and authorizes the use of the clinic's name in final reports written.

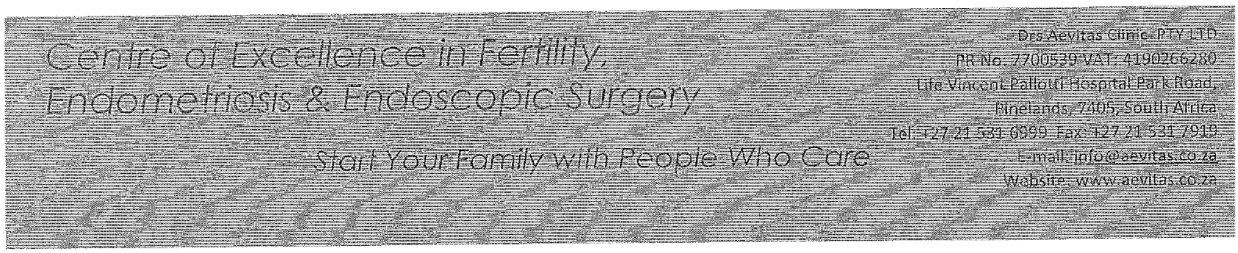
Kind regards,

Prof. TI Siebert

Dr. VA Hulme

Dr. G Hanekom

Date: 20 March 2020



Appendix J: Ethical approval

UNIVERSITEIT
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UNIVERSITY

Approval Notice

New Application

06/05/2020

Project ID :11627

HREC Reference No: S19/09/188

Project Title: The effects of co-culturing human embryos in one-step continuous culture media on assisted reproductive technology [ART] outcomes

Dear Miss Taryn Mclachlan

The **New Application** received on 08/04/2020 16:39 was reviewed by members of **Health Research Ethics Committee** via **expedited** review procedures on 06/05/2020 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Date: 06 May 2020

Protocol Expiry Date: 05 May 2021

Please remember to use your Project ID 11627 and Ethics Reference Number S19/09/188 on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Translation of the informed consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see *Forms and Instructions* on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Departement of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website https://applyethics.sun.ac.za/ProjectView/Index/11627

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Mrs. Brightness Nxumalo
HREC 2 Coordinator

National Health Research Ethics Council (NHREC) Registration Number:

REC-130408-012 (HREC1)•REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:

Appendix K: Datasheet

The information captured on the Microsoft Excel spreadsheet specifically designed for this study's statistical analyses.

General Cycle Information				Diagnostic Information			Ova/Fertilization Information				
Year	Patient code	ART Procedure	Group	Male	Female		# Ova	# MI/GV	# MII	# 2PN	FR (%)
				Diagnosis	Age (ova)	Diagnosis					

Blastulation Outcome Information											
D5 BT	D5 Blast (Grading)	# of Blasts (Obtained)				# of Blasts (Utilized)				# of Blasts (Remained)	
		Total	Good	Fair	Poor	Total	Good	Fair	Poor	Total	Good

Blastulation Outcome Information (continued)									ART Outcome Information		
# of Blasts (Remained)		D5 BDR				Proportion of Blasts			D5 ET	# ET	ET (Grading)
Fair	Poor	Total (%)	Good (%)	Fair (%)	Poor (%)	Good (%)	Fair (%)	Poor (%)			

ART Outcome Information (continued)					
# GS	#BB	IR (%)	CP	LB	MC

Data sheet key

General Cycle Information	
Year	Year in which ART cycles began.
Patient code	The unidentifiable patient code.
ART method	The method(s) of insemination utilized. **IVF** - In vitro fertilization **ICSI (ES)** - Intracytoplasmic sperm injection techniques utilizing ejaculated spermatozoa (traditional ICSI/PICSI/IMSI) **ICSI (TS)** – traditional ICSI utilizing testicular spermatozoa **Mixed** – In vitro fertilization and ICSI techniques
Group	Group assigned to cycle based on the embryo culture method employed. **0** - Group A utilizing embryo Culture Method A (embryos were individually cultured in sequential media) **1** - Group B utilizing embryo Culture Method B (embryos were co-cultured in 1-step continuous media)
Diagnostic Information	
Male – diagnosis	The diagnosis of the male producing the spermatozoa. **N** - Normozoospermia **O** - Oligozoospermia **A** – Asthenozoospermia **T** - Teratozoospermia **OA** - Oligo-asthenozoospermia **OT** - Oligo-teratozoospermia **AT** – Astheno-teratozoospermia **OAT** - Oligo-astheno-teratozoospermia **AZOO** – Azoospermia (testis biopsy sperm utilized for ova fertilization)
Female – Age (ova)	The age of the female undergoing the oocyte aspiration (years).
Female - Diagnosis	The diagnosis of female patient (based on the clinical findings). **MF** - Normal - Male factor **SD** - Normal – Sperm donor required (single female, same sex female couple) **OD** - Ovarian insufficiency, ova donor utilized **OA** - Ovarian insufficiency, autologous ova utilized **ENDO** - Endometriosis **AF** - Anatomical factor (ex: tubal factor) **EF** - Endocrine factor (ex: anovulation / PCOS) **RC** - Recurrent miscarriages **IDIO** - Idiopathic or unknown cause of infertility **S** – Surrogate required and utilized
Ova/Fertilization Information	

# Ova	The number of ova obtained from ova retrieval.
# GV/MI	The number of ova with germinal vesicles or in metaphase I at time of insemination.
# MII	The number of metaphase II oocytes at time of insemination.
# 2PN	The number of normally fertilized ova (2PN).
FR (%)	The fertilization rate (the proportion of mature ova that undergo successful fertilization 16-18 hours post insemination).
Blastulation Outcome Information	
D5 BT	The absence of presence of a day 5 blastocyst transferred. **0** – No blastocyst was transferred **1** – At least one blastocyst was transferred.
D5 Blast (Grading)	A list of the day 5 blastocysts' grades.
# of Blasts (Obtained)	The number of total, good-quality, fair-quality, poor-quality blastocysts obtained on day 5 of embryo culture.
# of Blasts (Utilized)	The number of total, good-quality, fair-quality, poor-quality blastocysts utilized on day 5 of embryo culture.
# of Blasts (Remained)	The number of total, good-quality, fair-quality, poor-quality blastocysts that remained on day 5 of embryo culture (following embryo transfer).
D5 BDR	The day 5 blastocyst development rate for the total, good-quality, fair-quality, poor-quality blastocysts.
Prop of day 5 Blasts	The proportion of good-quality, fair-quality, poor-quality blastocysts on day 5 of embryo culture.
ART Outcome Information	
D5 ET	The absence of presence of a day 5 embryo transfer. **0** – No embryo was transferred **1** – At least one embryo was transferred.
# ET	The number of embryos transferred.
ET (grading)	A list of the day 5 embryos' grades.
# GS	The number of gestational sacs observed via ultrasound.
#BB	The number of babies born.
IR (%)	The implantation rate (the percentage of transferred embryos that successfully implanted).
CP	The absence of presence of a clinical pregnancy. **0** – A clinical pregnancy did not occur. **1** – A clinical pregnancy did occur.
LB	The absence of presence of a live birth. **0** – A live birth did not occur. **1** – A live birth did occur.
MC	The absence of presence of a clinical pregnancy

	0 – A miscarriage did not occur. **1** – A miscarriage did occur.

Appendix L: Statistical analyses

Statistical analyses were performed to (i) ensure the two groups (Group A in which embryos were cultured using the embryo Culture Method A and Group B in which embryos were cultured using the embryo Culture Method B) were balanced and comparable, (ii) determine the blastulation outcomes for the two groups, and (iii) determine the ART outcomes for the two groups.

More specifically:

(A) To ensure that the two groups were balanced and comparable using descriptive statistics by:

1. Determining whether the groups had similar male diagnostic profiles.
2. Determining whether the groups had similar female diagnostic profiles.
3. Determining whether the groups had similar ART profiles.
4. Determining whether the groups utilized ova of similar ages.
5. Determining whether the groups utilized a similar number and maturity of ova.
6. Determining whether the groups had similar fertilization rates.
7. Determining whether the groups transferred a similar number of embryos.

(B) To determine the blastulation outcomes of the two groups by:

1. Calculating and comparing the blastocyst development rates.
2. Calculating and comparing the proportion of various-quality blastocysts.
3. Calculating and comparing the blastocyst attribution profiles.

(C) To determine the blastulation outcomes of the two groups by:

1. Calculating and comparing implantation rates.
2. Calculating and comparing clinical pregnancy rates.
3. Calculating and comparing live birth rates.
4. Calculating and comparing the miscarriage rates.

Next is a summary of the statical tests and calculations performed for this study. For more information regarding the abbreviations please refer to Appendix K:

A. Descriptive Statistics

1. Determining whether the groups had similar male diagnostic profiles.

Number and percentages of cases per male diagnoses:

Diagnoses	Group A		Group B		Total
	#	%	#	%	#
N	120	**65.22**	195	**66.10**	315
O	18	**9.78**	33	**11.19**	51
A	3	**1.63**	5	**1.69**	8
T	13	**7.07**	21	**7.12**	34
OA	1	**0.54**	11	**3.73**	12
OT	6	**3.26**	5	**1.69**	11
AT	1	**0.54**	1	**0.34**	2
OAT	3	**1.63**	1	**0.34**	4
AZOO (TB)	19	**10.33**	23	**7.80**	42
Total	184	**100**	295	**100**	479

Expected values:

Expected	Group A	Group B
N	121.00	194.00
O	19.59	31.41
A	3.07	4.93
T	13.06	20.94
OA	4.61	7.39
OT	4.23	6.77
AT	0.77	1.23
OAT	1.54	2.46
AZOO (TB)	16.13	25.87

Pearson Chi-square test ($P<0.05$): $p=0.3233$.

2. Determining whether the groups had similar female diagnostic profiles.

Number and percentages of cases per female diagnoses:

Diagnoses	Group A		Group B		Total
	#	%	#	%	#
MF	36	**19.57**	47	**15.93**	83
IDEO	36	**19.57**	34	**11.53**	70
SD	2	**1.09**	11	**3.73**	13
OD	59	**32.07**	118	**40.00**	177
OA	1	**0.54**	2	**0.68**	3
ENDO	17	**9.24**	24	**8.14**	41
AF	15	**8.15**	27	**9.14**	42
EF	11	**5.98**	23	**7.80**	34
RC	5	**2.72**	6	**2.03**	11
S	2	**1.09**	3	**1.02**	5
Total	184	**100**	295	**99.99**	479

Expected values:

Expected	Group A	Group B
MF	31.88	51.12
IDEO	26.89	43.11
SD	4.99	8.01
OD	67.99	109.01
OA	1.15	1.85
ENDO	15.75	25.25
AF	16.13	25.87
EF	13.06	20.94
RC	4.23	6.77
S	1.92	3.08

Pearson Chi-square test ($P<0.05$): $p=0.2244$.

3. Determining whether the groups had similar ART profiles.

Number and percentages of cases utilizing each category of ART:

Diagnoses	Group A		Group B		Total
	#	%	#	%	#
IVF	3	**1.63**	3	**1.02**	6
ICSI (ES)	123	**66.85**	221	**74.92**	344
ICSI (TS)	20	**10.87**	23	**7.80**	43
Mixed	38	**20.65**	48	**16.27**	86
Total	184	**100**	295	**100**	479

Expected values:

Expected	Group A	Group B
IVF	2.30	3.70
ICSI (ES)	132.14	211.86
ICSI (TS)	16.52	26.48
Mixed	33.04	52.96
Total	2.30	3.70

Pearson Chi-square test ($P<0.05$): $p=0.2873$.

4. Determining whether the groups utilized ova of similar ages.

Two sample t-test assuming equal variance

	Group A	Group B
Mean	**30.60**	**30.27**
Variance	26.94	30.95
Observations	184	295
Std Error	0.38	0.32
Std Dev	5.19	5.56
Pooled Variance	29.41	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	0.63	
P(T<=t) two-tail	**0.5261**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.75**	**0.64**
95% CI (Lower value)	**29.85**	**29.64**
95% CI (Upper value)	**31.35**	**30.91**

5. Determining whether the groups utilized a similar number and maturity of ova.

Total number of ova: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**12.68**	**13.08**
Variance	53.08	37.81
Observations	184.00	295.00
Std Error	0.54	0.36
Std Dev	7.29	6.15
Pooled Variance	43.67	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-0.64	
P(T<=t) two-tail	**0.5232**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**1.06**	**0.70**
95% CI (Lower value)	**11.63**	**12.38**
95% CI (Upper value)	**13.74**	**13.79**

Total number of mature ova: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**10.82**	**11.53**
Variance	39.91	31.20
Observations	184	295
Std Error	0.47	0.33
Std Dev	6.32	5.59
Pooled Variance	34.54	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-1.28	
P(T<=t) two-tail	**0.2002**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.92**	**0.64**
95% CI (Lower value)	**9.90**	**10.89**
95% CI (Upper value)	**11.74**	**12.17**

Total number of immature ova: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**1.86**	**1.55**
Variance	6.06	3.04
Observations	184.00	295.00
Std Error	0.18	0.10
Std Dev	2.46	1.74
Pooled Variance	4.20	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	1.62	
P(T<=t) two-tail	**0.1063**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.36**	**0.20**
95% CI (Lower value)	**1.51**	**1.35**
95% CI (Upper value)	**2.22**	**1.75**

6. Determining whether the groups had similar fertilization rates.

Two sample t-test assuming equal variance

	Group A	Group B
Mean	**82.25**	**80.83**
Variance	1158.50	300.20
Observations	184.00	295.00
Std Error	2.51	1.01
Std Dev	34.04	17.33
Pooled Variance	629.48	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	0.60	
P(T<=t) two-tail	**0.5483**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**4.94**	**1.98**
95% CI (Lower value)	**77.31**	**78.85**
95% CI (Upper value)	**87.18**	**82.82**

7. Determining whether the groups transferred a similar number of embryos.

Two sample t-test assuming equal variance

	Group A	Group B
Mean	**1.88**	**1.82**
Variance	0.26	0.18
Observations	184.00	295.00
Std Error	0.04	0.02
Std Dev	0.51	0.42
Pooled Variance	0.21	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	1.27	
P(T<=t) two-tail	**0.2035**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.07**	**0.05**
95% CI (Lower value)	**1.80**	**1.77**
95% CI (Upper value)	**1.95**	**1.87**

B. Blastulation Outcomes

1. Calculating and comparing the blastocyst development rates.

Total blastocyst development rate: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**40.70**	**53.96**
Variance	696.38	682.32
Observations	184.00	295.00
Std Error	1.95	1.52
Std Dev	26.39	26.12
Pooled Variance	687.71	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-5.38	
P(T<=t) two-tail	**<0.001**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**3.83**	**2.99**
95% CI (Lower value)	**36.87**	**50.97**
95% CI (Upper value)	**44.53**	**56.96**

Good-quality blastocyst development rate: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**4.45**	**11.97**
Variance	119.15	252.74
Observations	184.00	295.00
Std Error	0.80	0.93
Std Dev	10.92	15.90
Pooled Variance	201.49	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-5.64	
P(T<=t) two-tail	**<0.001**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**1.58**	**1.82**
95% CI (Lower value)	**2.87**	**10.14**
95% CI (Upper value)	**6.03**	**13.79**

Fair-quality blastocyst development rate: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**17.81**	**22.27**
Variance	380.27	314.89
Observations	184.00	294.00
Std Error	1.44	1.03
Std Dev	19.50	17.75
Pooled Variance	340.03	
Hypothesized Mean Difference	0.00	
df	476.00	
t Stat	-2.57	
P(T<=t) two-tail	**0.0104**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**2.83**	**2.04**
95% CI (Lower value)	**14.98**	**20.23**
95% CI (Upper value)	**20.64**	**24.31**

Poor-quality blastocyst development rate: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**18.44**	**19.80**
Variance	293.72	252.22
Observations	184.00	295.00
Std Error	1.26	0.92
Std Dev	17.14	15.88
Pooled Variance	268.14	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-0.88	
P(T<=t) two-tail	**0.3767**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**2.49**	**1.82**
95% CI (Lower value)	**15.96**	**17.99**
95% CI (Upper value)	**20.93**	**21.62**

2. Calculating and comparing the proportion of various-quality blastocysts.

Proportion of good-quality blastocysts: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**7.61**	**18.92**
Variance	248.70	551.19
Observations	161.00	281.00
Std Error	1.24	1.40
Std Dev	15.77	23.48
Pooled Variance	441.19	
Hypothesized Mean Difference	0.00	
df	440.00	
t Stat	-5.45	
P(T<=t) two-tail	**<0.001**	
t Critical two-tail	1.97	
95% CI Range (mean ±)	**2.44**	**2.75**
95% CI (Lower value)	**5.17**	**16.17**
95% CI (Upper value)	**10.06**	**21.68**

Proportion of fair-quality blastocysts: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**41.35**	**41.31**
Variance	1137.03	792.97
Observations	161.00	281.00
Std Error	2.66	1.68
Std Dev	33.72	28.16
Pooled Variance	918.08	
Hypothesized Mean Difference	0.00	
df	440.00	
t Stat	0.02	
P(T<=t) two-tail	**0.9873**	
t Critical two-tail	1.97	
95% CI Range (mean ±)	**5.23**	**3.30**
95% CI (Lower value)	**36.13**	**38.00**
95% CI (Upper value)	**46.58**	**44.61**

Proportion of poor-quality blastocysts: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**51.03**	**39.77**
Variance	1311.54	897.95
Observations	161.00	281.00
Std Error	2.85	1.79
Std Dev	36.22	29.97
Pooled Variance	1048.35	
Hypothesized Mean Difference	0.00	
df	440.00	
t Stat	3.52	
P(T<=t) two-tail	**0.0005**	
t Critical two-tail	1.97	
95% CI Range (mean ±)	**5.61**	**3.52**
95% CI (Lower value)	**45.42**	**36.25**
95% CI (Upper value)	**56.65**	**43.29**

3. Calculating and comparing the day 5 embryo transfer rates

Day 5 embryo transfer rates: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**88.04**	**95.76**
Variance	1058.45	407.49
Observations	184.00	283.00
Std Error	2.40	1.20
Std Dev	32.53	20.19
Pooled Variance	663.67	
Hypothesized Mean Difference	0.00	
df	465.00	
t Stat	-3.16	
P(T<=t) two-tail	**0.0017**	
t Critical two-tail	1.97	
95% CI Range (mean ±)	**4.72**	**2.36**
95% CI (Lower value)	**83.33**	**93.40**
95% CI (Upper value)	**92.76**	**98.12**

4. Calculating and comparing the blastocyst attribution profiles.

Total blastocysts obtained: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**3.66**	**5.13**
Variance	12.90	16.60
Observations	184.00	295.00
Std Error	0.26	0.24
Std Dev	3.59	4.07
Pooled Variance	15.18	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-4.03	
P(T<=t) two-tail	**<0.001**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.52**	**0.47**
95% CI (Lower value)	**3.14**	**4.66**
95% CI (Upper value)	**4.18**	**5.60**

Good-quality blastocysts obtained: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**0.48**	**1.26**
Variance	1.81	3.64
Observations	184.00	295.00
Std Error	0.10	0.11
Std Dev	1.35	1.91
Pooled Variance	2.94	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-4.84	
P(T<=t) two-tail	**<0.001**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.20**	**0.22**
95% CI (Lower value)	**0.28**	**1.04**
95% CI (Upper value)	**0.68**	**1.48**

Fair-quality blastocysts obtained: Two sample t-test assuming equal variance.

	Group A	Group B
Mean	**1.64**	**2.02**
Variance	4.64	3.31
Observations	184.00	295.00
Std Error	0.16	0.11
Std Dev	2.15	1.82
Pooled Variance	3.82	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-2.06	
P(T<=t) two-tail	**0.0395**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.31**	**0.21**
95% CI (Lower value)	**1.33**	**1.81**
95% CI (Upper value)	**1.95**	**2.23**

Poor-quality blastocysts obtained: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**1.54**	**1.85**
Variance	2.26	2.91
Observations	184.00	295.00
Std Error	0.11	0.10
Std Dev	1.50	1.71
Pooled Variance	2.66	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-2.06	
P(T<=t) two-tail	**0.0396**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.22**	**0.20**
95% CI (Lower value)	**1.32**	**1.65**
95% CI (Upper value)	**1.76**	**2.05**

Total blastocysts utilized: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**1.48**	**1.67**
Variance	0.51	0.32
Observations	184.00	295.00
Std Error	0.05	0.03
Std Dev	0.72	0.57
Pooled Variance	0.40	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-3.23	
P(T<=t) two-tail	**0.0013**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.10**	**0.07**
95% CI (Lower value)	**1.38**	**1.60**
95% CI (Upper value)	**1.58**	**1.74**

Good-quality blastocysts utilized: Two sample t-test assuming equal variance

	Group A	Group B
Mean	0.23	0.66
Variance	0.24	0.65
Observations	184.00	295.00
Std Error	0.04	0.05
Std Dev	0.49	0.81
Pooled Variance	0.50	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-6.49	
P(T<=t) two-tail	**<0.001**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.07**	**0.09**
95% CI (Lower value)	**0.16**	**0.57**
95% CI (Upper value)	**0.28**	**0.75**

Fair-quality blastocysts utilized: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**0.73**	**0.71**
Variance	0.58	0.56
Observations	184.00	295.00
Std Error	0.06	0.04
Std Dev	0.76	0.75
Pooled Variance	0.57	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	0.33	
P(T<=t) two-tail	**0.7430**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	0.11	0.09
95% CI (Lower value)	**0.62**	**0.62**
95% CI (Upper value)	**0.84**	**0.80**

Poor-quality blastocysts utilized: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**0.53**	**0.32**
Variance	0.56	0.35
Observations	184.00	295.00
Std Error	0.06	0.03
Std Dev	0.75	0.59
Pooled Variance	0.43	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	3.38	
P(T<=t) two-tail	**0.0008**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.11**	**0.07**
95% CI (Lower value)	**0.42**	**0.25**
95% CI (Upper value)	**0.64**	**0.39**

Total blastocysts remained: Two sample t-test assuming equal variance

	Group A	Group B
Mean	**2.17**	**3.46**
Variance	11.69	15.90
Observations	184.00	295.00
Std Error	0.25	0.23
Std Dev	3.42	3.99
Pooled Variance	14.28	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-3.62	
P(T<=t) two-tail	**0.0003**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.50**	**0.46**
95% CI (Lower value)	**1.67**	**3.00**
95% CI (Upper value)	**2.67**	**3.92**

Good-quality blastocysts remained: Two sample t-test assuming equal variance.

	Group A	Group B
Mean	**0.25**	**0.60**
Variance	1.10	1.98
Observations	184.00	295.00
Std Error	0.08	0.08
Std Dev	1.05	1.41
Pooled Variance	1.64	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-2.91	
P(T<=t) two-tail	**0.0038**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.15**	**0.16**
95% CI (Lower value)	**0.10**	**0.44**
95% CI (Upper value)	**0.40**	**0.76**

Fair-quality blastocysts remained: Two sample t-test assuming equal variance.

	Group A	Group B
Mean	**0.91**	**1.32**
Variance	3.66	3.24
Observations	184.00	295.00
Std Error	0.14	0.10
Std Dev	1.91	1.80
Pooled Variance	3.41	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-2.32	
P(T<=t) two-tail	**0.0207**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.28**	**0.21**
95% CI (Lower value)	**0.63**	**1.11**
95% CI (Upper value)	**1.54**	**1.53**

Poor-quality blastocysts remained: Two sample t-test assuming equal variance.

	Group A	Group B
Mean	**1.01**	**1.54**
Variance	2.19	3.15
Observations	184.00	295.00
Std Error	0.11	0.10
Std Dev	1.48	1.77
Pooled Variance	2.78	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-3.35	
P(T<=t) two-tail	**0.0009**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**0.21**	**0.20**
95% CI (Lower value)	**0.80**	**1.34**
95% CI (Upper value)	**1.22**	**1.74**

C. ART Outcomes

1. Calculating and comparing implantation rates.

Two sample t-test assuming equal variance

	Group A	Group B
Mean	**36.23**	**38.36**
Variance	1806.97	1540.20
Observations	184.00	295.00
Std Error	3.13	2.28
Std Dev	42.51	39.25
Pooled Variance	1642.55	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-0.56	
P(T<=t) two-tail	**0.5762**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**6.16**	**4.49**
95% CI (Lower value)	**30.07**	**33.87**
95% CI (Upper value)	**42.40**	**42.86**

2. Calculating and comparing clinical pregnancy rates.

Two sample t-test assuming equal variance

	Group A	Group B
Mean	**46.74**	**54.92**
Variance	2502.97	2484.26
Observations	184.00	295.00
Std Error	3.69	2.90
Std Dev	50.03	49.84
Pooled Variance	2491.44	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-1.74	
P(T<=t) two-tail	**0.0819**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**7.25**	**5.71**
95% CI (Lower value)	**39.48**	**49.21**
95% CI (Upper value)	**53.99**	**60.62**

3. Calculating and comparing live birth rates.

Two sample t-test assuming equal variance

	Group A	Group B
Mean	41.85	47.12
Variance	2446.84	2500.17
Observations	184.00	295.00
Std Error	3.65	2.91
Std Dev	49.47	50.00
Pooled Variance	2479.71	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	-1.13	
P(T<=t) two-tail	**0.2604**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	7.17	5.73
95% CI (Lower value)	34.67	41.39
95% CI (Upper value)	49.02	52.85

4. Calculating and comparing miscarriage rates.

Two sample t-test assuming equal variance

	Group A	**Group B**
Mean	**10.33**	**9.15**
Variance	931.04	834.31
Observations	184.00	295.00
Std Error	2.25	1.68
Std Dev	30.51	28.88
Pooled Variance	871.42	
Hypothesized Mean Difference	0.00	
df	477.00	
t Stat	0.42	
P(T<=t) two-tail	**0.6723**	
t Critical two-tail	1.96	
95% CI Range (mean ±)	**4.42**	**3.31**
95% CI (Lower value)	**5.90**	**5.84**
95% CI (Upper value)	**14.75**	**12.46**

Appendix M: Plagiarism report

THE EFFECTS OF CO-CULTURING HUMAN EMBRYOS IN ONE-STEP CONTINUOUS CULTURE MEDIA ON BLASTULATION AND ASSISTED REPRODUCTIVE TECHNOLOGY OUTCOMES

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