The effect of varying decreased sperm concentrations on embryogenesis using Embryoscope time lapse monitoring.

By:

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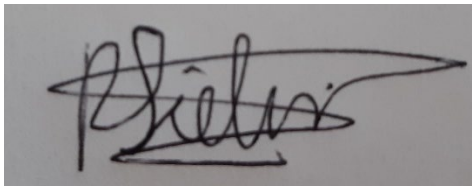
Date: 10 August 2021

DECLARATION

I, Rizqa Siebritz, declare that the contents of this thesis represents my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



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ABSTRACT:

Background:

Assisted Reproductive Technology (ART) has become one of the predominant optional technologies for couples struggling with natural conception. Male infertility is a major cause of ART failure. With the use of the Embryoscope, embryos can be better monitored *in vitro*, improving the selection criteria for embryo transfer.

Aim:

The aim of this study was to utilize the embryoscope to investigate the effects of decreased sperm concentrations (<10 million/ml), motility and abnormal morphology on ovum fertilisation and embryonic development up until the blastocyst stage.

Method:

60 patients with a combined total of 586 ova which underwent ICSI were monitored using the Embryoscope time lapse monitoring system, from injection until blastocyst stage. Each patients' semen biochemical analysis and morphology was performed. Ovum were then collected and monitored using the Embryoscope time lapse monitoring system. This data was then analysed and statistically compared using Statistica.

Results:

Of the sixty participants,11 had a low sperm concentration (group A), 16 a normal concentration (group B) and 33 a high concentration (group C). Greater than 80% of all ovum reached metaphase II allowing them to be injected. However, those with a reduced sperm count in group A had a significantly lower percentage of ovum reaching metaphase II compared to those with normal and high sperm concentration (66% vs 79% vs 77% $p<0.014$). In addition, 53% of ovum in group A reached the final blastocyst stage which was lower compared to group B with 71% and group C with 67% ($p<0.133$). While those with a lower sperm concentration had a decrease in ova development in the first two stages of embryogenesis there was however no significant difference between the growth rates of ova in all groups. In multivariate analysis sperm motility and morphology did not influence ova survival or growth rates.

Conclusion:

The findings of this study show that participants with low sperm counts had significantly lower fertilization and lower percentages of ovum reaching the blastocyst stage of development. There was however no significant difference in the rate of growth, and abnormal morphology and motility had no impact on the results.

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- Finally, to my family and husband for the unwavering support and grit when I needed to pull late nights and long off days to get my writing done. My Ohana. Forever blessed.

DEDICATION:

This is dedicated to all those who have supported me through this long Masters process. It has taught me many things like academic and personal growth. It has also reminded me time and time again that hard work and dedication are always the key to success.

Mom and Dad = I made it

GLOSSARY:

- Blastocyst – A thin-walled hollow structure in early embryonic development that contains a cluster of cells called the inner cell mass from which the embryo arises. The outer layer of cells gives rise to the placenta and other supporting tissues needed for foetal development within the uterus while the inner cell mass cells give rise to the tissues of the body.

- Blastomeres – One of the cells produced by cleavage of a fertilized ovum.

- Cytokinesis - is the physical process of cell division, which divides the cytoplasm of a parental cell into two daughter cells.

- Fertilization – The penetration of the ovum by the spermatozoon and combination of their genetic material resulting in the formation of a zygote.

- Hatching – Around the end of the fifth day the embryo frees itself from the enveloping zona pellucida. Through a series of expansion-contraction cycles the embryo bursts the covering zona. The rhythmic expansions and contractions result in the embryo bulging out of and emerging from the rigid envelope. This "first birth" is called hatching.

- Infertility – A disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse.

- Mitosis - cell division that results in two daughter cells each having the same number and kind of chromosomes as the parent nucleus, typical of ordinary tissue growth.

- Morula – A solid ball of cells resulting from division of a fertilized ovum, and from which a blastocyst is formed.

- Oocyte – An egg cell of the animal ovary; in humans, one oocyte matures during the menstrual cycle.

- Ovum – The female reproductive cell or gamete of animals and humans.

- Ova – Plural of Ovum.

- Pronuclei - Either of a pair of gametic nuclei, in the stage following meiosis but before their fusion leads to the formation of the nucleus of the zygote.

- Zona Pellucida - The strong membrane that forms around an ovum as it develops in the ovary. The membrane remains in place during the egg's travel through the fallopian tube. To fertilize the egg, a sperm must penetrate the thinning zona pellucida. That is not completely accurate. You can better say: After 5-6 days the embryo is hatching through the Zona Pellucida to permit implantation in the uterus.

LIST OF ABBREVIATIONS:

AIDS – Acquired Immune Deficiency Syndrome

AMH – Anti-Mullerian Hormone

ART – Assisted Reproductive Technology

DNA – Deoxyribonucleic acid

DOR – Diminished Ovarian reserve

ET – Embryo Transfer

HIV – Human Immunodeficiency Virus

ICSI – Intra Cytoplasmic Sperm Injection

IVF – In Vitro Fertilization

IUI – Intra Uterine Insemination

MAR Test – Mixed Antiglobulin Reaction Test

PCOS – Polycystic Ovary syndrome

STI – Sexually Transmitted Infections

t2 – 2 blastomeres

t4 – 4 blastomeres

t8 – 8 blastomeres

tMp – Morula

tB – Early blastocyst

TESE – Testicular Sperm Extraction

TLS – Time Lapse Systems

WHO – World Health Organisation

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CHAPTER 1

1.1 Introduction and Significance of research

The rights of South Africans with respect to reproduction has shifted from focussing on an individuals' right to avoid reproduction (abortion and contraceptives), to understanding the rights of individuals to have access to treatment for infertility with the assistance of assisted reproductive technology (ART). In the ground-breaking constitutional court decision of AB vs Minister of Social Development 2017 3 BCLR 267 (CC) it was suggested that reproductive rights exist only if the person who is physically involved in the process is claiming the rights. Since this judgment, ART has become the way forward for couples struggling with natural conception (Van Niekerk, 2017).

ART is a term used to describe all procedures where gametes are handled outside the body but does not include procedures in which spermatozoa are used such as intrauterine insemination (IUI). *In vitro* fertilisation (IVF) is the first and most common of the ART procedures, however ART also includes: embryo transfer (ET), gamete intra fallopian transfer, zygote intra-fallopian transfer or pronucleate, intra cytoplasmic sperm injection (ICSI), round nuclei injection or spermatid injection and assisted hatching. The most common are, IVF &ET and ICSI (Begum, 2008).

ART is a timely and expensive procedure which many couples undergo to realise their dream of bearing a child. The effects of ART and its risk factors are explained by Squires and Kaplan (2007). This article describes the potential effects of procedures that require human egg and sperm manipulations with the use of fertility, embryo culture and transfer of a growing foetus. Most studies examining ART have demonstrated no added risk for the development of problems for children born after ART. However, problems may include congenital abnormalities, early delivery, genetic abnormalities, growing delays with mental and behavioural health and disabilities. (Squires & Kaplan, 2007). Therefore, it is clear that there is a risk for opposing perinatal outcomes in IVF pregnancies, however it is noted that the majority of the children born following IVF will have a good outcome (Babooa & Chen, 2015).

To date the primary risk factors contributing to poor outcomes in ART offspring is prematurity and low birth weight. Currently, 51% of ART births in the United States have increased risk of early health problems, disability and multiple births (Squires & Kaplan, 2007). The ART

research community have created different tools to better understand ovum growth and selection. Techniques such as embryo grading and embryo time lapse monitoring have been at the forefront of this research (Babalan *et al*., 2011). The Embryoscope is an instrument developed to monitor the growth from fertilization through all stages of embryonic development until the moment of implantation. The Embryoscope assists embryologists and researchers to monitor ovum growth and helps compare the impact of low sperm concentrations, abnormal morphology and other abnormalities on firstly; the ratio of eggs being fertilised as well as secondly; the amount of eggs going through the stages of development and lastly identifying any change in rate of development (Brugh *et al*., 2003).

This current research project would therefore be significant as no investigations utilising the Embryoscope to examine the effects of decreased sperm concentrations on the rate of embryo development have been done in South Africa. Male infertility has become a growing risk factor to couples around the world and this research project aims to utilise the Embryoscope to examine the effects of decreased sperm concentration on the rate of embryo development in South Africa. Many researchers believe doctors sell false hope in the form of ART and this study could help identify sources of male infertility which have a greater risk of incomplete implantation and therefore failure of ART (Straussburger *et al*., 2000). This study will also analyse the effect of sperm concentration as most previous studies has focussed on the correlation of fertilization with morphology (Lundin *et al*., 1991). This research could therefore aid in improving methodologies in patients identified as having decreased success rate with the use of certain ART methods.

1.2 Aim

The aim of this study was to evaluate, utilizing the Embryoscope and its time lapse and videography information as a tool, the effects of decreased sperm concentrations (<10 million), abnormal morphology and motility on ovum fertilisation and embryonic development up until the blastocyst stage.

1.3 Objectives

- To compare fertilisation rates of male participants who have sperm concentrations of <10 million/mL with participants who have sperm concentrations of between 10 – 50 million/mL and >50 million/mL

- To compare the above groups A, B and C and the rate of embryonic development according to the following stages: 2 pronuclei, 2 cell, 4 cell, Morula and Blastocyst
- To correlate the above groups A, B and C and the number of growth stages reached, helping to understand if there is a specific growth stage where development is halting.
- To examine the effects of abnormal morphology and motility on fertilisation rates and embryonic development in contrast to sperm concentration.

CHAPTER 2

2.0 Background and Literature Review

2.1 Infertility

The International Committee for Monitoring Assisted Reproductive Technology, World Health Organisation (WHO), describes infertility as a disease of the reproductive system which is described as the failure to achieve a clinical pregnancy after one year or more of regular unprotected sexual intercourse (Niederburger, 2009; World Health Organisation Department of Reproductive Health and Research, 2010). Infertility is sub-divided into two main categories: primary and secondary infertility. Primary infertility refers to couples who have not been able to conceive after at least 1 year of having unprotected sex while secondary infertility refers to couples who have been able to achieve a previous pregnancy, but are now unable to conceive after at least 1 year of having unprotected sex (Sharma, 2017).

Globally, 15% of couples are reported to be affected by infertility however information regarding rates of male infertility is lacking and has therefore not been accurately reported (Argarwal *et al*., 2015). The WHO has stated that the ratio for the amount of couples worldwide whom encounter problems in conceiving a first child is one in eight, with this ratio being one in six when couples attempt to conceive a second child (World Health Organisation Department of Reproductive Health and Research, 2010). 3% of women remain childless involuntarily, with the number of parous women not being able to conceive as many children as they would like to, at 6% (Jungwirth *et al*, 2016; Szkodziak et al., 2016). Male infertility has been the factor for 50% of involuntary childless couples. A woman may compensate for the infertility parameter the male individual is lacking; thus, infertility usually manifests if both individuals have reduced fertility (Kuma & Singh, 2015)

Approximately 8% of males seek medical assistance for fertility related problems (Argarwal *et al*., 2015). Research by the World Health Organisation noted that fifteen percent of couples (48.5 million globally) were unable to have children (World Health Organisation Department of Reproductive Health and Research, 2010). The largest population of male infertility originates in central and eastern Europe and Australia. However, as male infertility is stigmatised in African countries these figures may not be accurate (Mascarenhas *et al*., 2012). The United Nations have undertaken global fertility studies between 2015 -2020 which have shown a decrease in total fertility see Figure 1(United Nations, 2017). Global reports of infertility have

increased over the past decade and affects both males and females. Much research has been done to find out the cause of this increase and possible reasons have included, an increase in the prevalence of obesity, exposure to cell phone radiation and certain endocrine disorders (Levine *et al*., 2013; Sharma, 2017).

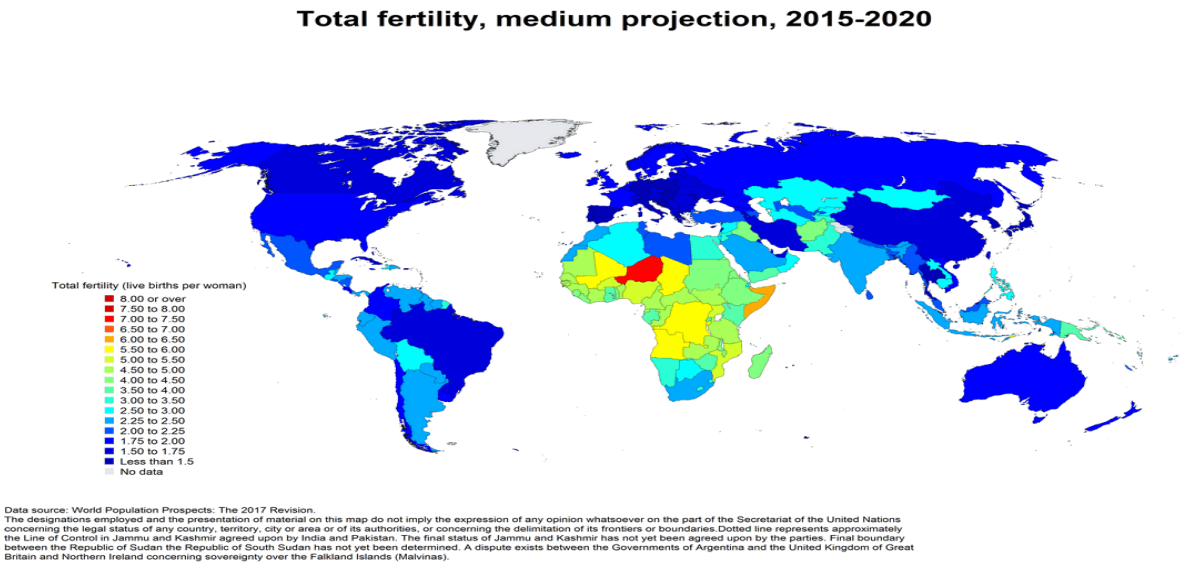

Figure 1: Total fertility, medium projection, 2015 – 2020 (United Nations, 2017)

2.2 Causes and treatments of infertility

Female infertility is caused by a number of factors such as anovulation, endometriosis, cervical insufficiencies; uterine defects (congenital or acquired); changes in the frequency and duration of the menstrual cycle; fallopian tube damage or abnormalities, defects of the peritoneum and poor lifestyle or dietary choices (Anwar & Anwar, 2016). Low sperm production, function or blockages that prevent the delivery of sperm are some of the major causes of male infertility. Other causes include injuries, chronic health problems, illnesses, lifestyle choices and other factors (Sharpe, 2012). Common lifestyle causes include increased alcohol intake, weight, smoking and stress (Mulgund *et al*., 2015).

Some male signs and symptoms of infertility can include erectile dysfunction, ejaculation of small amounts of semen and reduced desire for sexual activity. Men can also experience pain and swelling in the testicular genital areas with abnormal breast enlargement, decreased body hair and increased respiratory infections (Jungwirth, 2012). In 30-40% of cases, no causal factor for the male associated infertility is found (idiopathic male infertility). Males can present with no previous history of disease affecting fertility and on physical examination, endocrine

analysis, genetic and biochemical laboratory testing, findings are normal. When a semen analysis is performed however, abnormalities on the spermiogram can be seen. Endocrine abnormalities due to environmental pollution, genetic and epigenetic disorders or reactive oxygen species can be assumed to be causes of idiopathic male infertility (Jungwirth *et al*., 2016).

Reactive oxygen species (ROS) is a generic term used to describe an array of derivatives of molecular oxygen that occur normally in the body. Increased formation of ROS can lead to molecular damage (oxidative stress) by overwhelming the protective mechanisms in the lipid layers of the spermatozoa plasma membrane (Sanocka & Kurpisz, 2004). Furthermore, it can directly or indirectly cause changes in the spermatozoas DNA through the activation of sperm endonucleases and caspases. Oxidative stress is caused by an individuals' lifestyle (obesity), testicular defects, infection, alcohol consumption and smoking (tobacco contains nicotine, tar, carbon monoxide and heavy metals all toxicological mechanisms) (Takeshima *et al.*, 2018).

DNA fragmentation can occur either in single strand or double stranded DNA and can affect all cell components, reducing sperm motility and mitochondrial activity. This damage can be repaired by the human oocyte and embryo; however, the repair ability decreases with advanced maternal age. Spermatozoa with increased DNA fragmentation are meant to fall into apoptosis however when they do not, they can adversely affect the embryos development and implantation (Takeshima *et al.*, 2018). The meta-analysis of Zhao et al (2014) showed that increased sperm DNA damage was significantly associated with pregnancy [combined relative risk (RR): 0.81; 95% confidence interval (CI): 0.70–0.95; P = 0.008] and miscarriage (combined RR: 2.28; 95% CI: 1.55–3.35; P < 0.0001).

Once couples suspect they may be infertile, they would be referred to medical experts for investigation. The investigation includes: a semen analysis with the use of the World Health Organisation (WHO) guidelines (World Health Organisation Department of Reproductive Health and Research, 2010) and a clinical examination by a specialist in Reproductive Medicine or a Urologist. Further analysis could include a hormonal investigation with the determination of follicle stimulating hormone, testosterone and luteinizing hormone levels, microbiological assessment for the presence of urinary tract infections, sexually transmitted diseases and male accessory gland infections is important. Furthermore, genetic testing including an extensive family history and karyotype analysis; ultrasonography and if necessary, a testicular biopsy are all important investigations (Palermo *et al*., 2014; Barak, 2016).

The WHO has standardised semen analysis with the publication of the WHO laboratory manual for the examination and processing of Human Semen (World Health Organisation Department of Reproductive Health and Research, 2010). The standardised semen analysis comprises of a sperm concentration and total sperm count, volume and pH, sperm motility, sperm vitality, sperm morphology round cells and leucocytes, and sperm antibodies (Kliesh, 2014; World Health Organisation Department of Reproductive Health and Research, 2010). The WHO guidelines using specific parameters classifies male infertility status by differentiating them into the following categories (Table 1): Oligozoospermia:<15 million/mL; Asthenozoospermia:<32% progressive motile sperm; Teratozoospermia:<4% normal forms and if all three abnormalities occur concurrently it is defined as Oligo-asteno-teratozoospermia (OAT) syndrome (Jungwirth *et al*., 2012; Zhu *et al*., 2016; World Health Organisation Department of Reproductive Health and Research, 2010).

Table 1: Lower reference limits for semen characteristics (World Health Organisation Department of Reproductive Health and Research, 2010)

Parameter	Lower reference limit
Semen volume (ml)	1.5 (1.4–1.7)
Total sperm number (10^6 per ejaculate)	39 (33–46)
Sperm concentration (10^6 per ml)	15 (12–16)
Total motility (PR+NP, %)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0)
Other consensus threshold values	
pH	≥7.2
Peroxidase-positive leukocytes (10^6 per ml)	<1.0
MAR test (motile spermatozoa with bound particles, %)	<50
Immunobead test (motile spermatozoa with bound beads, %)	<50
Seminal zinc (μmol/ejaculate)	≥2.4
Seminal fructose (μmol/ejaculate)	≥13
Seminal neutral glucosidase (mU/ejaculate)	≥20

Once a diagnosis of male infertility has been made, there are treatment options that could be followed. If there are abnormalities on the semen analysis, a clinician should refer the male to a specialist in Reproductive Medicine or a Urologist, whilst anatomic variance or obstruction would require surgical evaluation and treatment (Lindsay & Vitrikas, 2015). Other treatment options include: lifestyle changes, hormonal treatment, infection treatment, retrograde ejaculation stimulation treatment, immunologic infertility treatment with corticosteroids, testicular hypothermia devices, sperm processing for inter-uterine insemination and the last option is in-vitro fertilisation (IVF) (Aziz, 2012). If none of the above treatments improve the semen analysis parameters, ART is a valid option.

2.3 Sperm Concentration

Sperm concentration is defined as the number of sperm per millilitre in a semen sample and is typically determined by counting sperm in a counting chamber. According to the WHO laboratory manual for the examination and processing of human semen 5th edition, the lower reference limit for sperm concentration is 15 x 10^6 spermatozoa/mL (Rage: 12 - 16 x 10^6 spermatozoa/mL) (World Health Organisation Department of Reproductive Health and Research, 2010), see table 1. The Sperm Count is the total number of sperm in an entire ejaculation and is determined when the sperm concentration is multiplied by the total semen volume received. The lower reference limit for a sperm count is 39 x 10^6 spermatozoa per ejaculate (Range: 33 - 46 x 10^6 spermatozoa per ejaculate), see table 1. The presence of previous ejaculate that had not been previous cleared out correctly (partial or total), and the duration of abstinence before the analysis can all affect the total number of sperm found in the semen ejaculate (World Health Organisation Department of Reproductive Health and Research, 2010).

The number of Sertoli cells in the testes and the time since the last ejaculation (abstinence) are used to determine a males' sperm count. Sertoli cells are somatic cells of the testis, which are required for the formation of the testis and assist in spermatogenesis. These cells facilitate the movement of germ cells to the growing spermatozoa by direct contact (Hess & Luiz, 2005) The number of Sertoli cells are determined in the early stages of development (Sharpe, 2012), and after puberty, sperm begin to be produced continuously in the testes, with each sperm taking approximately 10 weeks to mature. The rate at which sperm are used up is determined by the frequency of ejaculations, and the balance between supply and demand (Sharpe, 2012).

Sperm concentration is the single best predictor of conception and, because the correlation between concentration and conception is so significant, some researchers have proposed that other parameters may not play a significant role (Poon, 2011). A concentration of above 50 million sperm per millilitre will saturate the female reproductive tract and higher counts do not provide additional benefit (Poon, 2011; Guzick *et al*., 2001; Li *et al*., 2019). While sperm concentrations of less than 50 million per millilitre have a negative impact, concentrations of less than 15 million per millilitre could affect fertility. Men with low sperm concentrations report fertilization rates of 30%, while those with average sperm concentrations have a 60 – 80% fertilization rate (Prajamati & Solanki, 2011).

A low sperm concentration should not be viewed as a definitive diagnosis. In a large 2001 analysis, sperm concentrations of below 13.5 million/ml were considered a strong indication of infertility. In this study semen samples from 765 infertile men and 696 men from fertile couples were evaluated at 9 different locations across America. The results showed that there were no specific criteria that leads to infertility. It was also noted that the shape and appearance of sperm (known as morphology) appeared to be an important measurement for discriminating between fertile and infertile men. (Gurick *et al*., 2001).

In contrast, an earlier study done by Burris et al in 1988 aimed to define the minimal number of sperm needed for conception. In this study semen characteristics of men with isolated hypogonadotropic hypogonadism (IHH) were investigated. 22 of 24 men (92%) proved to be fertile and were able to achieve a total of 40 pregnancies with a mean sperm concentration of 16.7 ± 4.0 X 10^6/ml. They concluded that men with IHH who have sperm concentrations below the lower limit of 16.7 ± 4.0 X 10^6/ml can still initiate conception (Burris *et al*., 1988). Despite having all the other parameters on a semen analysis report, the most important measurement is the semen concentration (Poon, 2011).

Figure 2 demonstrates the effects of semen concentration on pregnancy rates and shows that it is the most important factor determining the ability to naturally achieve pregnancy. Lower concentrations have lower pregnancy rates compared to those with a concentration of 50 x 10^6/ml

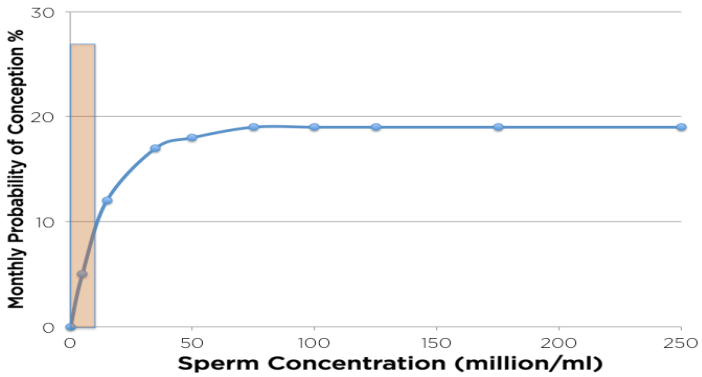

Figure 2**:** Concentration and Pregnancy (Poon, 2011)

2.4 Other parameters affecting male infertility

Using the semen analysis report to determine the probability of pregnancy is impossible without understanding other factors that could affect the couples' ability to conceive**.**

2.4.1. Sperm motility

Sperm motility refers to the movement of the sperm in a semen sample (Sharp, 2012). Normal sperm motility is considered when a minimum of 40% of the sperm in the sample are motile (Sharpe, 2012). Sperm motility plays an important role in men's reproductive health and natural conception including a successful pregnancy, cannot happen if sperm are unable to move. When assessing motility, progressive motility is also taken into consideration. This assesses the number of sperm moving from one area to another, as opposed to twitching or going in circles. In this case, if the total number of sperm in the sample that are progressively motile is 32% (Range: 31 – 34%), it is considered acceptable see table 1 (World Health Organisation Department of Reproductive Health and Research, 2010).

2.4.2 Sperm morphology

Sperm morphology is considered a non-specific parameter, as the semen of a typical fertile male is often composed of morphologically abnormal spermatozoa (Figure 3) (Menkveld *et al*., 2011). However, over the past three decades the criteria for the assessment of sperm morphology has become very strict and the WHO considers >4% normal morphology as adequate (World Health Organisation Department of Reproductive Health and Research, 2010). However, the use of sperm morphology as an infertility indicator is controversial. Researchers believe the morphology considered normal is subjective and varies greatly between technologists (Niederberger, 2011). The predictive value of sperm morphology therefore remains controversial see figure 4, it shows that monthly probability of contraception stays relatively constant between 10 to 20% with an increasing sperm morphology % from 4 – 12%. Even though the acceptable limit according to WHO is 4% (see table 1). The contraception probability decreases below 10% when the morphology % is 2% or less. (Bartoov *et al*., 2002; Poon, 2011; Menkveld *et al*., 2011).

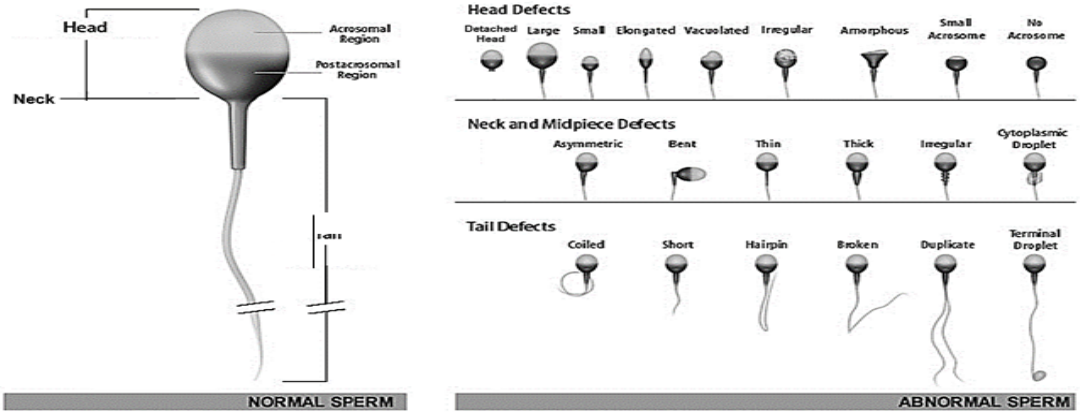

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Figure 3: Sperm Morphology (World Health Organisation Department of Reproductive Health and Research, 2010)

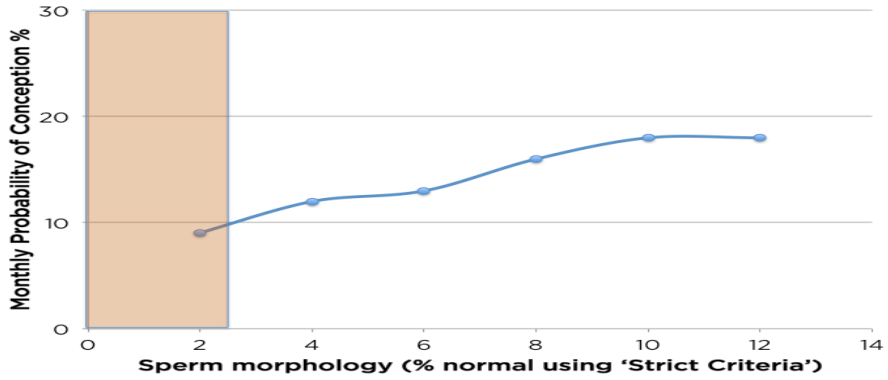


Figure 4: Sperm morphology vs Conception (Poon, 2011)

2.4.3 Age and Infertility

Sperm quality deteriorates as men get older but generally has no effect until men reach the seventh decade of life (Yerebasmaz *et al*., 2017). Men are noted to have a gradual change in fertility and sexual functioning as they get older. However, no clinical maximum age has been proven whereby men can no longer have children. Testes tend to get smaller and softer, and sperm morphology (shape) and motility (movement) decrease together with an increased risk of genetic defects. Older men develop illnesses and lifestyle habits that over time begin to adversely affect their sexual and reproductive function. A healthy lifestyle over the years can allow men to experience healthy reproductive and sexual functioning (Bartoov *et al*., 2002; De Kluiver *et al*, 2017).

2.5 *In Vitro* Fertilisation vs Intra Cytoplasmic Sperm Injection:

In Vitro Fertilization (IVF) means “fertilisation in glass”. It is a technique known as assisted reproduction in which the egg and sperm are fertilised outside the body to form an embryo. Embryos are inserted into the uterus to implant and result in a pregnancy. Louise Brown was the first baby to be born using IVF and was born in 1978 in the United Kingdom (Kovacs, 2014). IVF was initially developed to assist in the treatment of infertility which was as a result of blocked or damaged fallopian tubes however, this has expanded to include a wider variety of infertility problems. The 2010 Nobel Prize in Physiology or Medicine was awarded to Dr R.G. Edwards for the development of human embryo IVF, which has dramatically improved the treatment of many types of infertility (The Noble Assembly of Karolinska Institute, 2010).

IVF is a procedure whereby sperm and eggs are combined outside of the body in a laboratory dish and allowed to fertilise naturally. On the other hand, ICSI which uses a similar principle to IVF, requires medical personnel to inject the spermatozoa directly into the egg to bring about fertilization. Severe male infertility management has been assisted dramatically by ICSI and live birth rates are superior to those with other non-donor treatments (Kovacs, 2014).

Couples who have had poor or no fertilisation during standard IVF or are trying to overcome male infertility are recommended to try ICSI instead. ICSI was first used in 1992 and was an alternative option for males diagnosed with severe male infertility, which includes: poor sperm motility (slowly moving sperm), a low sperm count, poor sperm morphology (abnormal shaped sperm), an obstruction that prevents sperm release (such as vasectomy) and/or anti-sperm antibodies (antibodies which may inhibit sperm function and is produced by the male himself) (Jungwirth *et al*, 2002). At Cape Fertility Clinic ICSI is offered to all those undergoing assisted reproductive services to increase the chances of egg fertilisation.

2.6 The process of *In-Vitro* Fertilisation (IVF)

There are five basic steps to IVF. The process begins with: Stimulation of ovaries, also known as super ovulation, whereby medications are used to boost egg production. The ovarian stimulation protocol was adapted from the standard operating procedures (SOPs) used at Cape Fertility Clinic following the guidelines established by SASREG (Society to Specialists in the field of Reproductive medicine). The procedure begins with day 1 of the menstrual cycle (first day of the menstrual period), on day 3 of the cycle the female participant will begin with fertility injections of Gonal-F®, Pergoveris® (Merck, South Africa (Pty) Ltd) or Menopur®

(Ferring Pharmaceuticals, South Africa (Pty) Ltd taken daily. On day 8 the first ultrasound scan was done; ultrasound scans are done to monitor the amount of eggs present and to estimate which would be the best day for egg collection. Additionally, from day 8 Cetrotide® (Merck, South Africa (Pty) Ltd) medication is administered daily. On day 10 and 12 the second and third ultrasound is performed. If on day 12 the follicles are of a sufficient size a hormone injection is administered in the evening and the eggs are collected 48 hours later.

Secondly, egg collection, is performed with minor surgery known as follicular aspiration. A reproductive specialist with the use of a transvaginal ultrasound probe removes the egg from the egg-containing follicles within the female ovaries. A needle attached to the ultrasound probe which is monitored using ultrasound, is carefully guided into each follicle and the contents (follicular fluid, granulosa, and oocytes) are aspirated into a warm plastic test tube (Gardner, 2007).

Thirdly, insemination and fertilization are carried out. Insemination is the process of fertilising ova collected, with sperm. This is done by transferring eggs into an incubator in a dish containing culture medium (resembles fluids found in the fallopian tubes and uterus) where they are kept at specific conditions that support their growth requirements. The dishes which contained the eggs are then placed into an incubator which is temperature and gas controlled (Hoeger *et al*., 2013). A few hours after eggs are retrieved, sperm is placed in the culture medium with the eggs (technique known as IVF) or individual sperm are injected into each mature egg in a technique (ICSI).

Fourthly, using embryo culture, the eggs are returned to the incubator or placed in time lapse monitoring systems, where they remain to develop until blastocyst stage. Periodically over the next few days, the dishes are inspected in order to assess the development of the embryos. Sixteen to eighteen hours after the eggs have been inseminated/injected they are examined by the embryologist for signs of fertilisation. Fertilisation is considered to have been achieved when two pronuclei or two polar bodies are detected. After five days, once the eggs have developed to the blastocyst stage (a fertilised embryo that has more than eighty cells in an inner fluid filled cavity and a small cluster of cells known as an inner cell mass) the embryo can be transferred into the female (Gardner, 2007).

Finally, in step number five, called embryo transfer, the blastocysts are placed into the uterus which is done by using a catheter (Kovacs, 2014). Because IVF and ICSI is a costly procedure most individuals opt to transfer more than one embryo for greater chance of pregnancy. This

however leads to an increase in multiple pregnancies. A developing embryo can be affected by many factors before leading to pregnancy such as: whether the embryo developed well in the laboratory, the females' state of mind and health and the embryos ability to attach to the endometrium (Jones *et al*., 1998; Cetin *et al*., 2010).

2.7 Light vs Dark Time Lapse Systems (TLS)

Prior to time lapse embryologists had to remove embryos from the incubator daily in order to assess cleavage and morphology. However, embryos do not tolerate removal from optimal culturing conditions, and this therefore limited the required number of observations. As this was a problem for embryologists', time lapse monitoring systems (TLS) were created. The first TLS was manufactured in 1997 by utilising a IX-70 inverted microscope (Olympus Optical Co., Tokyo, Japan), equipped with a Perspex environmental chamber which was maintained at 37 degrees Celsius and a humidified atmosphere with 5% carbon dioxide (Wu *et al*., 2016). To exclude extraneous light, the chamber was fully covered during recording, and occurred during the darkness hours. A purpose-built switching box turned the microscope lamp on for 5 seconds every minute (Mio & Maeda., 2008).

With this technology, embryos are monitored without having to be removed from the incubator. The incubator has a built-in camera which takes a picture of the embryos at pre-set timed intervals. Upgraded video software depicts the development of each embryo from snapshot to snapshot taken by the camera. This technology allows the embryologist to monitor the embryos growth without removing it from the incubator with the cleavages and morphological changes of the embryo being monitored from inception to blastocyst stage.. The first researchers to report on the early events of human embryonic growth and development were Payne et al (Payne *et al*., 1997). Mio et al followed this with an explanation of the kinetics of the events up until the blastocyst stage (Mio & Maeda., 2008).

Various time lapse systems are currently used with two of the most widely used technologies being the Primo Vision (VitroLife) and Embryoscope (FertiliTech) systems which utilise bright light field technologies. The EEVA (Early Embryonic Viability Assessment, Auxogyn) systems from Merck uses dark field technology (VitroLife, 2015). All 3 systems take pictures at 5 to 20-minute intervals using a digitally inverted microscope. The images are then displayed on a computer screen using custom image acquisition and are then played in sequence in short films that can be fast forwarded and re-wound for better detailed analysis of the embryos. Light

TLS uses a light microscope to project the image of the embryo whereas dark TLS uses special dark field illumination to better outline the cell membranes (Kovacs, 2014).

2.8 The Embryoscope

The Embryoscope produced by VitroLife is the most used TLS for the observation of embryo development while maintaining stable embryo culture conditions. An Embryoscope is the combination of an incubator, a microscope with an integrated camera and advanced software. This software has been used in more than three hundred thousand patient cases since 2009 (Kovacs, 2014). Embryos are individually cultured with a maximum of twelve per multi-dish. A multi-dish is specifically made for the Embryoscope and is known as an Embryoslide. They are moved one by one into the field of view of the built-in microscope at each of the image acquisitions. It uses low intensity red LED illumination (635nm) with <0.5 seconds per image light exposure. The Embryoscope can follow six of these dishes (maximum seventy-two embryos) simultaneously. It evaluates the embryos using seven different focal points and takes pictures every 12 to 20 minutes (VitroLife, 2015).

The built-in camera is used to monitor oocysts in the closed incubation environment from fertilization to embryo transfer. This information is transferred to the Embryoscope server so that the information can be accessed from a computer. The incubator is an extremely stable culture environment, with the temperature being regulated with direct heat contact. The incubator air is continuously purified by a filter, and controlled by carbon dioxide and oxygen, this helps remove any contaminants (Meseguer *et al*., 2012). The Embryoscope uses time lapse systems to provide an image which embryologists use to visually follow the development of the embryos, and to choose the one with the best implantation potential.

Using the embryoscope and its annotations feature, one can record the times of events occurring during embryo development. They constitute the base on which the embryo evaluation can be performed using time-lapse monitoring in the IVF laboratory. The use of time-lapse technology to detect developmental abnormalities is immense. The annotations facilitate the immediate overview of the course of embryo development, by annotating cell divisions the Embryoscope software builds a division chart for the Embryologist to assess. Embryologists can, based on morphologic characteristics and annotated timings, score the embryos at various stages of development. The benefits of the Embryoscope is that it improves embryo selection; culture conditions and embryo handling as well as providing the parents with more information about their embryos and its development (Rubio *et al*., 2014).

Earlier cleaving of embryos has been previously reported to have a better chance for development into blastocyst and implantation. Also, it should be noted that the decision on which embryo to transfer should not be rushed and is better to monitor the embryo until blastocyst stage and perform transfers on day five. Many fertility centres use extended culture to the blastocyst stage even though this practice is labour intensive and increases the cost of embryology procedures (Kirkegaard *et al*., 2013).

2.9 Embryonic development stages

Once oocytes are removed and injected with spermatozoa, they are placed in the Embryoscope and monitored at different stages of development. Initially a normal looking ovum should have cytoplasm, a single polar body, an appropriate zona pellucida (ZP) thickness and proper perivitelline space (PVS) see figure 5. The completion of meiosis has not yet completed at the point of fertilization with oocytes arrested in metaphase of meiosis II until fertilisation is completed. (Magli *et al*., 2012). The embryo then stores unneeded complement of genetic material in a second polar body that is eventually expelled. This is the point at which an oocyte can now be called an ovum (with female haploid chromosomes inside). The two haploid nuclei derived from the sperm and oocyte and contained within the egg are referred to as pronuclei. This is the first stage that can be seen and monitored using the Embryoscope and the appearance of the two haploid pronuclei will be stage one of development timed (Magli *et al*., 2012).

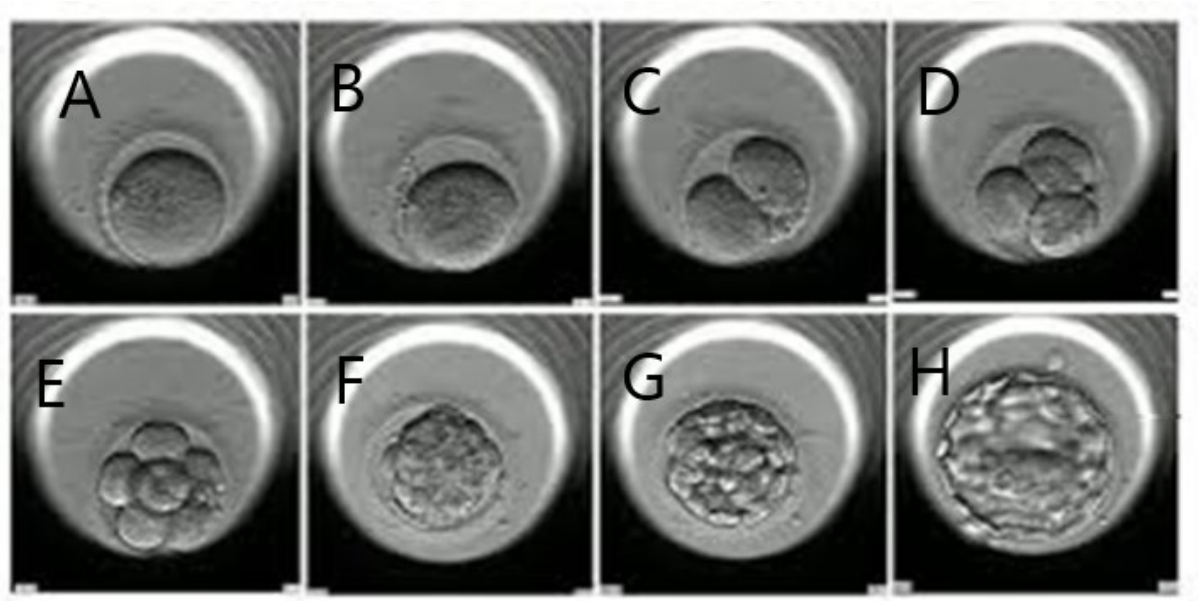


Figure 5: Growth stages under Embryoscope – A: Ovum, B: 2 Pronuclei; C: 2 Cell; D: 4 cell; E: 6-8 cell; F: Morula; G: Early blastocyst; H: Late blastocyst (Vitrolife, 2015)

The two pronuclei de-condense, expand, and replicate their DNA in preparation for mitosis. The pronuclei then move toward each other, their nuclear envelopes disintegrate, and the male- and female-derived genetic material intermingles. The completion of fertilization results in a single-celled diploid zygote with all the genetic instructions it needs to develop into a human. This is followed by the cleavage phase - The conceptus is now made up of two to sixteen cells (known as blastomeres) but has no blastocystic cavity and the zona pellucida can still be easily recognised. The separation of embryos at stage 2 cell, 4 cell, 8 cell and Morula (sixteen to 32 cells) are observed and annotated using the Embryoscope and stored as video data footage for easy comparison.

The last and final stage in the incubator is the blastocyst stage (Magli *et al*., 2012). The conceptus is now a blastocyst which is characterised by a hollow mass of cells with a blastocystic cavity. The blastomeres now segregate into an internal inner cell mass and an outer trophoblast (Yamada & Takakuwa, 2012). This stage is also annotated using the Embryoscope and recorded for comparative studies.

Mitosis in blastomeres should produce two equally sized daughter cells, when the division is asymmetric (one blastomere larger than the other) the next generation will inherit less than half the amount of cytoplasm from the parent blastomere which may lead to a defective lineage in the embryo. An example is 4 to 8 cell embryos with equal cell size have shown to have lower multinucleation and aneuploidy rate and increased implantation rates (Scott *et al*, 2008; Magli *et al*, 2012). Irregularities in embryo development has been studied using morpho-kinetics and time-lapse systems. Morpho-kinetic annotations of cell divisions during the transition between the pronuclear and the eight-cell stage indicated that direct cleavage of embryos from one to three and from two to five blastomeres are negatively associated with successful implantation (Wirka *et al*, 2020).

A qualified embryologist can distinguish whether the eggs are passing through the stages of development and can use the TLS to monitor ovum growth and time the changes in growth over the period of 5 days before egg transfer. With the use of time lapse monitoring it is possible to analyse patients' embryos and compare embryos of males with a sperm count of <10 million and compare them to groups with higher counts. This can be used to determine if

sperm counts influence the number of eggs fertilised and embryonic development (Kovacs, 2014).

2.10 Previous research on the use of the Embryoscope to predict embryo development

In the beginning stages of embryonic research, it was found that there was a marked variation in timings of polar body extrusion and pronuclear formation. These timings differed significantly amongst different oocytes formed after day three of monitoring via the time lapse system (Payne *et al*., 1997). Lemmen et al evaluated 102 2 Pronuclei (2PN) oocytes using time-lapse monitoring and demonstrated that rates of growth and cytoplasmic events were similar to that obtained in standard incubators. This study concluded that the camera monitoring system had no impact on the embryos as they were growing (Lemmen *et al*., 2008).

Further studies monitored embryos for differences in developmental kinetics. By tracking the development of 242 two stage pronucleate embryos Wong et al, were able to evaluate three predictive parameters which were seen to differ in blastocyst development: firstly, the time to cytokinesis (mean 14.3 ± 6 min), secondly, the time between first and second mitosis (mean: 11.1 ± 2.2 hrs) and third, the time between second and third mitosis (mean: 1 ± 1.6 hrs). It was noted there was no significance in results seen as there was no difference between the three events between the population of eggs studied (Wong *et al*., 2010)

Continuing from this study, Meseguer *et al.*, using continuous kinetic studies demonstrated the use of monitoring the kinetics of embryo development on implantation potential. Two hundred and forty-seven time-lapse embryo observations were monitored in which the implantation success rate for all transferred embryos was known. The cleavage of all embryos from one to three cells (very short time as a two-cell embryo; [< 5 hrs]), uneven blastomeres at the two-cell stage (>25% size difference) and multi-nucleation at the four-cell stage were all noted as predictors of the embryo having a decreased chance of implantation. These results suggested that they be used as an exclusion criterion for egg transfer (Meseguer *et al*., 2011).

This initial study by Meseguer *et al* (2012) began a basis for specific exclusion criteria for embryos. These exclusion criteria (direct cleavage from one to three cells) were later used as the basis for other studies including one such as Dal Canto *et al* (2012) in which four categories were established. This study monitored 71 cycles of ART (22 standard IVF and 49 ICSI). 459 zygotes were subjected to time-lapse analysis. 8 cell stage death occurred in 157 (34.2%),

whilst 151 (32.9%) reached blastocyst stage of development. Cleavage times showed that first cleavage in standard IVF was considerably slower than that of ICSI (28.6+/- 2.6h versus 37.6 +/- 3.7h, P = 0.02). Afterward cleavage was identical, particularly at 8 cell standard IVF cleavage time was 60.8 +/-7.9h and ICSI cleavage was at 60.90 +/-10.6h. This research concluded that embryos at two-cell stage with uneven blastomeres as well as embryos cleaving directly from one to three cells were unlikely to become blastocysts or to become a blastocyst with good morphology (Dal Canto *et al*., 2012; Aguilar *et al*., 2014; Azzarello *et al*., 2012; Chamayou *et al*., 2013).

This study supported previous observations and helped identify an association between the rates of growth at the beginning of embryogenesis and the formation of blastocysts. The authors suggested that this could predict if embryos would end up becoming viable blastocysts for embryo transfer. A further study evaluated the use of automated time-lapse analysis system in determining its value in the prediction of blastocyst formation. The results of this investigation demonstrated that the system had a positive predictive value: of 54% and a negative predictive value of 86% (Conaghan *et al*., 2013). The value of automated time lapse monitoring was confirmed by work performed on establishing the predictive analysis of the time to first cytokinesis, to 2 pronuclei phase and the time to three-cell stage. In this study it was noted that the lack of cleavage to three cell stage from cytokinesis was indicative of a high-quality blastocyst (Kirkegaard *et al*., 2013; Hererro *et al*., 2013).

Mizobe *et al* monitored the Embryoscope and its efficacy as an incubator. The study monitored the changes in developmental patterns of embryos using the Embryoscope specifically. The results showed that the Embryoscope is of great value when determining which embryo should be selected for implantation. It monitors all the eggs developmental stages and their timeline, which is directly linked to an embryo's growing ability and implantation possibility. A high-quality embryo can be selected using the embryoscope when all these factors are considered (Mizobe *et al*., 2014). The Embryoscope, assists the standardizing of morphokinetic variable analysis by providing uninterrupted culture, for up to 6 days if required (Cruz *et al.*, 2011; Pribenszky *et al*., 2010). It has also demonstrated that culture in this environment improves the incidence of pregnancy and shown that aneuploid and euploid embryos have differing morphokinetic variables under IVF conditions (Campbell *et al*., 2013)

More recent studies of time-lapse systems have indicated that the timing of the cleavage is not the only factor to monitor but also the time of each cell division. Blastomeres should divide equally and in synchronicity, studies observed only two, four and eight cell embryos but

neglected to examine three, five, six, seven or nine cell embryos, which is an indication that blastomeres are not dividing in synchronicity (Scott *et al*., 2008; Lemmen *et al*., 2008; Wong *et al*., 2010; Meseguer *et al*., 2011; reviewed by Kirkegaard *et al*., 2013).

Evidence of paternal influence on embryonic genome activation and early embryonic development, blastocyst formation and implantation have been noted (Tesarik *et al*., 2004; Neyer *et al*., 2015; Sacha *et al*., 2020). The impact of male infertility on the morpho-kinetic patterns studied previously remains unclear, defects in the centrosome of the spermatozoa can contribute to early morpho-kinetic changes as many genes are not activated until later in the embryogenesis stages. Tesarik *et al* (2004) found that sperm DNA fragmentation has no adverse impact on early embryo cleavage morphology after ICSI but may negatively impact the embryos ability for implantation. In contrast Esbert *et al* (2018) studied showed the association between sperm DNA fragmentation and slower cell division. The quality of the spermatozoa injected into the ovum can also affect the morpho-kinetic parameters and the completion of embryogenesis.

In the study by Neyer *et al* (2015) embryo morpho-kinetics and blastocyst development were compared against male factor infertility and recurrent implantation failures. Two thirds of the embryos evaluated did not fulfil the morpho-kinetic criteria, noting that good quality spermatozoa tend to yield more high-quality blastocysts regardless of morphokinetic criteria. It has been shown that patient- and treatment-related factors such as maternal age, infertility indication, sperm quality, stimulation regimes and day of transfer as well as fertilization method, culture media, oxygen concentrations and other variations between laboratories, influence embryo kinetics and viability (Campbell *et al.*, 2013; Hampl & Stepan, 2013; Lemmen *et al*., 2008, Marion *et al.*, 2021).

More recent studies have investigated the impact the embryologist have on the outcomes on ICSI regarding ART its specific protocols, timings and conditions required for the embryo to develop. In a study by Maggiulli *et al* (2020) acknowledged that no report addressed issues such as operator skills and experience. The study showed that the mean time from induction of ovulation to oocyte denudation was 39.3 +/- 6.4 min, with blastulation rate per cohort if inseminated oocytes was 34 +/- 2.9%. No association was found for denudation and ICSI timings and operators. This is important to note as all staff are trained professionals with several years of experience at Cape Fertility and it is highly unlikely that the operators influenced the outcomes.

2.11 Conclusion

The above articles clearly demonstrate the utilisation of the embryoscope in the monitoring of embryogenesis has significant value in assisting in the choice of the most suitable embryo or transfer. However, most studies have examined and focussed on female egg progression and associated abnormalities while the effects of male abnormalities are scarce. Although ART and ICSI aim to overcome spermatozoa defects, clarity on the effects of motility, concentration and morphology in these procedures remains unclear. The aim of this study was therefore to analyse the effects of male sperm concentration and other abnormalities on blastocyst development.

CHAPTER 3

3. Research design and methodology

3.1 Study design

This was a prospective study in which the fertilisation and embryonic development was compared between three groups of male participants with specific sperm concentrations. The groups were compared for ovum development rate and completion of embryogenesis with the use of the embryoscope and its annotation abillity. The effects of motility and morphology as an independent covariant factor and their effects on concentration related to ovum embryogenesis completion was also examined and compared between the three groups. The study took place at Cape Fertility in Claremont, Cape Town. The three groups were separated by concentration and were identified as: Group A: concentration =/< 10 million/ml, Group B: concentration 10 – 50 million/ml and Group C: concentration >50 million/ml.

3.2 Inclusion and Exclusion criteria

All couples who had decided to have ART with the use of ICSI at the Cape Fertility Clinic from January – October 2018 were included in the study. Participants were required to sign a consent form (Appendix B) which outlined the procedure and included an option of utilising the Embryoscope to monitor their ovum development. Couples were excluded from the study if they chose not to monitor their ovum with the Embryoscope function and patients who signed the consent form but did not accept the terms of being part of the research section. Patients who opted to use a sperm donor were also excluded.

3.3 Specimen collection

3.3.1 Semen collection and analysis

Semen samples were collected by masturbation once patients had abstained from sexual activity for 2 to 7 days according to WHO criteria. Samples were collected in a private room near the laboratory for easy transportation and, if collected at home, were required to reach the laboratory within 1 hour and kept as close to body temperature as possible (World Health

Organisation Department of Reproductive Health and Research, 2010). Once the semen sample arrived at the laboratory it was kept in an incubator for 45 minutes to an hour to liquefy. Each sample required a 16ml press top tube, 4 glass frosted end slides and a plastic pasteur pipette. The slides were prepared for 1) Wet preparation 2) Mixed Antiglobulin Reaction (MAR) test (Done only on participant samples with a motility percentage of greater than forty) 3) and 4) Morphology.

a. Mixed Antiglobulin Reaction (MAR) test

The Mixed Antiglobulin Reaction (MAR) test was performed using a kit manufactured by FertiPro (from Beernem, Belgium) and was performed according to the manufacturer instructions. A drop of sperm was added to a drop of sensitised blood and a drop of anti-IgA. Thereafter the slides were checked for moving spermatozoa with the antigen attached. One hundred spermatozoa were counted, and the result was expressed as a percentage. >50% spermatozoa with a bead attached either to the head, neck or tail was considered positive (Paschke *et al*.,1994).

b. Morphology

The morphology slides were air dried and stained with a diff quick stain manufactured by Medion Grifols Diagnostics AG (from Dudingen, Switzerland) and evaluated against the World Health Organisation criteria for sperm morphology (World Health Organisation Department of Reproductive Health and Research, 2010). Sperm consists of a head, neck and tail (midpiece and principal piece). Normal spermatozoa morphology was seen if the tail and head had a normal appearance, the head should be smooth oval shaped and the acrosomal region comprising of 40-70% of the head and contain no vacuoles.

The midpiece was considered normal if it was slender, regular and about the same length as the sperm head and the major axis of the midpiece was aligned with the major axis of the sperm head. If there was no residual cytoplasm and when it exceeded one third of the sperm head, it was considered abnormal. Normal spermatozoon had a principal piece which was uniform in length, thinner than the midpiece, and was approximately 45 nm long (about 10 times the head length). Although it may be looped back on itself (see Figure 3), there were no sharp angles which would indicate a flagellar break. The following morphological categories were considered as abnormal (see Figure 3). These included: head defects: abnormal shaped with a tapered, round, or amorphous shape with large vacuoles and/or double heads

Neck and midpiece defects: asymmetrical insertion of the midpiece into the head and or thick. Principal piece defects include broken, smooth bends, coiled and or short. All borderline forms were considered abnormal and if >/= 5% of the spermatozoa had normal morphology it was considered a normal semen sample.

c. Sperm Motility

Sperm motility within semen was assessed as soon as possible after liquefaction of the sample, to limit the effects of dehydration, pH and temperature and processed within 30 minutes to one-hour following ejaculation. Ten microliters of semen were placed on a glass slide and covered with a 10 x 10mm glass coverslip. Thereafter, a minimum of 200 spermatozoa within five high power fields were counted and classified according to three motility categories: progressive motility, non-progressive motility and immotility. These categories were calculated as percentages and compared to the normal range of >40% motile spermatozoa (progressive and non-progressive) and >32% progressive spermatozoa (World health Organisation Department of Reproductive Health and Research, 2010).

d. Sperm Concentration

A sperm concentration was determined for each sample with the use of a Neubauer counting chamber. A well-mixed semen sample was aspirated, allowing no time for the spermatozoa to settle out of suspension and dispensed into a fixative, then vortexed for ten seconds at max speed and loaded onto the Neubauer chamber. The Neubauer chamber was left to stand horizontally for four minutes at room temperature allowing the immobilized cells to sediment onto the grid. The Neubauer has two square grids, on the first grid two hundred spermatozoa were counted, making sure to count the number of rows it took to get to two hundred spermatozoa. This count was then repeated on the other square grid, the difference between the two grids were checked for acceptability (Figure 6 for grid).

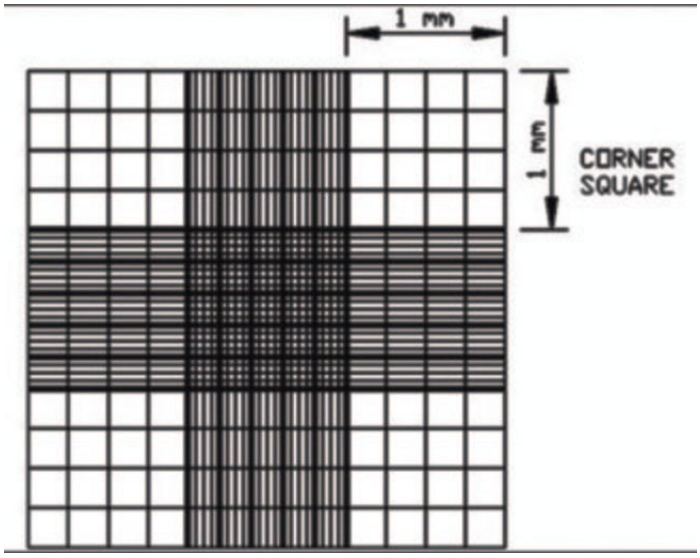


Figure 6: Improved Neubauer chamber (Electron Microscopy Sciences, 2019)

If the count was determined as acceptable, the concentration was then calculated. The concentration of spermatozoa in semen is calculated as follows: their number (N) divided by the volume, i.e. the volume of the total number (n) of rows examined for the replicates (20nl each for grids 4, 5 and 6), multiplied by the dilution factor. That is, C = (N/n) × (1/20) × dilution factor. The normal range is >12 spermatozoa per millilitre (World Health Organisation Department of Reproductive Health and Research, 2010). This semen concentration would be used to distinguish the three groups used in this study. The WHO laboratory manual for the examination and processing of human semen was created to maintain a consistent standard for semen analysis to obtain the most accurate results possible around the world. The guideline makes special note of the guidelines for patients to adhere to in order to control the outcome of the results examples: abstinence of two to seven days.

3.3.2. Ovarian stimulation and collection by Ovum pick up method:

Stimulation of ovaries, also known as super ovulation, whereby pharmaceutical treatments were used to boost egg production was performed according to WHO criteria (World Health Organisation Department of Reproductive Health and Research, 2010). The ovarian stimulation protocol was adapted from the standard operating procedures (SOPs) used at Cape Fertility Clinic following the guidelines established by SASREG (Society to Specialists in the field of Reproductive medicine). The procedure begins with day 1 of the menstrual cycle (first day of the menstrual period), on day 3 of the cycle the female participant will begin with fertility injections of Gonal-F®, Pergoveris® (Merck, South Africa (Pty) Ltd) or Menopur® (Ferring Pharmaceuticals, South Africa (Pty) Ltd) taken daily. On day 8 the first ultrasound

scan was done; ultrasound scans are done to monitor the amount of eggs present and to estimate which would be the best day for egg collection. Additionally, from day 8 Cetrotide® (Merck, South Africa (Pty) Ltd) medication is administered daily. On day 10 and 12 the second and third ultrasound is performed. If on day 12 the follicles are of a sufficient size a trigger injection is administered in the evening.

On the 14th day ovum pick-up was performed next door to the sterile laboratory room. This was optimal for the preparation of the collecting tubes with follicular fluid. On the day before the *In Vitro* Fertilization (IVF), preparations were made for both the pick-up and insemination. This included the preparation of a five well petri dish with 0.5ml growth enzyme and 1.5ml oocyte wash. A press top tube filled with industrial sperm wash and press top tube filled with flush media were kept in a 37-degree incubator with 5-6% carbon dioxide and 5% oxygen for ovum pick-up the following day.

The ovum pick-ups were done in theatre with an aspiration pump and needle. The pre-packed sterilised needle was flushed with oocyte flush medium and then inserted into the female patients' ovary. During the aspiration, the follicular fluid flowed through the pump tubing and into a 10ml press top tube. Once the tube was filled to 8ml it was placed into a warming block in the embryology screening room which was easily accessible by a window attached to the theatre room. The tube was removed from the warming block and screened for oocytes. Once an oocyte/s was found under the microscope it was transferred to a small petri dish filled with oocyte wash and placed in the screening incubator. The dish was then moved to the equilibrating incubator to compensate for the loss of gas.

The oocytes were examined for the presence of metaphase II. Metaphase II is the second stage of meiosis II. The two daughter cells produced during the first meiotic division line up and the spindle draws their chromosomes to opposite poles for separation. At this phase, the egg is ready for fertilisation.

3.4 *In Vitro* Fertilization (IVF) procedure: Intra-Cytoplasmic Sperm Injection (ICSI) Procedure

To increase the chances of fertilisation the oocytes undergo intra cytoplasmic sperm injection. The procedure starts by breaking the tail membrane. This procedure was done by spinning down the sperm sample which has been mixed with 1ml sperm wash. It was centrifuged for 10 minutes at 1000rpm and the supernatant was removed with a clean pipette and discarded.

Sperm buffer was then added to the pellet and incubated for an hour. The sperm were then added to a Polyvinyl Pyrrolidone (PVP) strip that was placed in the centre of a small petri dish. Using a micro-needle, the sperm tails were nicked off one by one. Once tail-less the micro-injector was used to suck up the sperm head and inject it into the oocytes which have been transferred into a 5 well petri dish with the addition of fertilisation media and denuding acid which had been removed to the outer ovum layer for easier sperm head injection (Magli *et al*., 2008).

Following an overnight incubation, the oocytes were analysed for fertilisation. The oocytes are placed on the screening microscope and cleaned with a flexi pipette. Once cleaned the oocytes can easily be evaluated for the presence of pronuclei which if present are left in the same culture medium from day one till the day of transfer into the patient.

3.5 Embryo Monitoring

Once the eggs were in the incubator, they would be monitored over a period of 5 days using the Embryoscope monitoring system. This with the use of the time lapse functionality of the Embryoscope will allow for the categorisation of each fertilised egg according to stages of development. The exact time each stage of development was reached was annotated. The stages include stage 1: 2 Pronuclei (2PN) where fertilization is confirmed; stage 2: 2 blastomeres (t2); stage 3: 4 blastomeres (t4); stage: 8 blastomeres (t8); stage 5: morula (tMp) ; stage 6: early blastocyst (tB). The annotation system (figure 7) integrates an automatic image analysis feature, it automatically detects cell division and morphological events and estimates division timings and morphological parameters. All 6 stages mentioned above were detected manually using the annotation system, it the tabulates the timed results (figure 8) (Vitrolife, 2019).

This information was collected from the monitoring of the ovum during embryogenesis and was plotted onto a chart of time versus stage reached. A graph for each participant was created and compared and correlated with sperm concentration, morphology, and age. Fertilization was assessed by determining how many ova had reached stage 2 above.

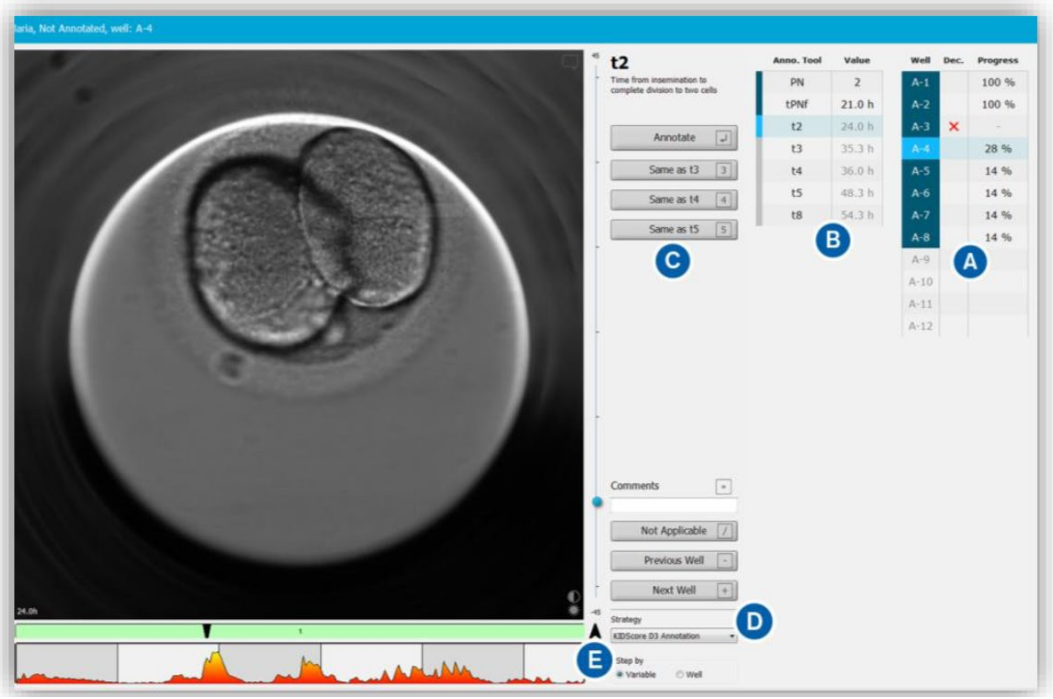


Figure 7: Embryoscope annotation system (Vitrolife; 2019)

Anno. Tool	Value
PN	***2***
t2	***25.5 h***
t3	***38.1 h***
t4	*39.6 h*
t5	***50.5 h***
tB	*110.4 h*
ICM	***A***
TE	***A***

Figure 2: Example of variable values estimated by the Guided Annotation tool.
*Estimates within the confidence threshold are depicted in **bold italic** (A) and those outside the threshold are depicted in italic (B).*

Figure 8: Example of annotation (Vitrolife, 2019)

3.6 Ethics

The ethical approval for this study was obtained from the Cape Peninsula University of Technology Ethics committee (***CPUT/HW-REC 2018/H3)*** (Appendix C) and approval for the study was given by the Cape Fertility clinic to utilise existing data (Appendix A). Each participant was required to read and sign a consent form allowing the data to be used for the

research (Appendix B point 11). In addition, to maintain confidentiality, each participant was given a unique number which was different to the allocated laboratory number from the Cape Fertility Clinic. This ensured the name and personal details of the participant remained anonymous. All the tests and procedures described in the study were routinely performed on the participants' including the Embryoscope and Ovum Monitoring.

3.7 Statistical analysis

According to the World Health Organisation (Agarwal et al., 2015), 15% of couples suffer from infertility. Using the formula below it was estimated that a total of 44 participants would be required to be recruited.

Detect r = 0.4 in a population given alpha = 0.05 and beta = 0.2 (Power = 0.8)
Thus, H0: r = 0,
H1: r ≠ 0

t tests - Correlation: Point Bi-Serial model

Analysis:	A priori: Compute required sample size	
Input:	Tail(s)	= Two
	Effect size |ρ|	= 0.4
	α err prob	= 0.05
	Power (1-β err prob)	= 0.8
Output:	Noncentrality parameter δ	= 2.8949875
	Critical t	= 2.0180817
	Df	= 42
	Total sample size	= 44
	Actual power	= 0.8073726

All participants' data recorded was a combination of the raw data of a semen analysis result with that of biochemical tests done by the Cape Fertility Clinic and the addition of the Embryogenesis graph created by the researcher with the use of the Embryoscope. Annotation data was collected until the total number of 60 participants was gathered as per the calculation above. The results of the semen biochemical analysis, morphology and the annotation data obtained from the Embryoscope was entered onto a Microsoft Excel spread sheet and the three groups were compared using statistical methods. In the end

60 participants' raw data were used with a total of 586 ovum monitored and time data recorded, each participant had between 3 -12 ovum incubated.

A p value of less than 0.05 was significant. To analyse the demographical data to achieve a p value, the one-way Annova statistical method was used. The one-way analysis of variance (ANOVA) is a statistical test which determines if statistically significant differences exist between the means of two or more independent (unrelated) groups (Rutherford, 2011).

To analyse the clinical data different statistical methods were used to achieve a p value. For survival data (measuring the ovum survival during embryogenesis) the Kaplan–Meier estimator was used, it is also known as the product limit estimator, is a non-parametric statistic used to estimate the survival function from lifetime data (Chan, 2004). For comparative group data the Chi-square test was used, is intended to test how likely it is that an observed distribution is due to chance (Ugoni & Walker, 1995). Age, motility, and morphology were analysed as co-variables using cox-regression.

CHAPTER 4

4. Results

4.1 Patient demographics

Of the sixty participants recruited onto the study 11 had a low sperm concentration (group A), 16 had a normal concentration (group B) and 33 had a high concentration (group C). There was no significant difference in the age of the participants, and none had any sexually transmitted diseases or HIV. There was no significant difference in the age of the female donors, or the quality and number of eggs collected (all: $p>0.05$). 41 out of 60 participants had a morphology examination performed. The remaining participants did not have morphology completed due to insufficient sample. All participants had a total motility test performed. Group B and C have greater than normal average total motility scores of greater than 48%, Group A has a low average total motility score of 15%. The demographic details of the 3 groups of participants are recorded in Table 2.

Table 2: Patient demographics

Demographic / Parameter	**Mean and Range**				**P value**
	Total participants	**Group A**	**Group B**	**Group C**	
Number of participants	60	11	16	33	n/a
Mean Concentration (million/ml)	68.85 (0 – 297)	1.98 (0 – 5.25)	33.50 (13.75 – 50)	110.11 (55 – 297)	<0.001
Mean Male Participant Age	40 (28 – 54)	40 (28 – 49)	38 (30 – 50)	41 (29 – 54)	.511
Number of participants using a Female Donor	36	7	8	21	n/a
Mean Female Recipient Age (years)	38 (27 – 49)	36 (28 – 44)	37 (27 – 46)	40 (31 – 39)	0.076
Mean Female Donor Age (years)	26 (17 - 33)	25 (20 - 29)	27 (22 - 31)	25 (17 - 33)	0.557
Ova monitored	586	107	154	325	n/a
Number of ova per participant	13.30 (5 – 31)	14.82 (7 – 31)	12.38 (5 – 21)	13.24 (6 – 27)	0.551
Number of participants who had Sperm	42 (out of 60)	2 (out of 11)	13 (out of 16)	27 (out of 33)	n/a

morphology test performed					
Number of participants who had Total motility performed	59 (out of 60)	11 (out of 11)	16 (out of 16)	32 (out of 33)	n/a
Percentage of total motility	47%	15%	49%	55%	n/a
Positive BHCG value after ICSI (%)	37.9%	45.5%	21.4%	42.4%	n/a

4.2 Ova reaching metaphase II after ovum hyper-stimulation

There was a significant difference in the percentage of mature ova collected a between the three groups ($p < 0.001$). Although all three groups had high returns on egg viability (>80%) the number of mature ova collected for Group A was significantly lower than those for Groups B and C. (Figure 9).

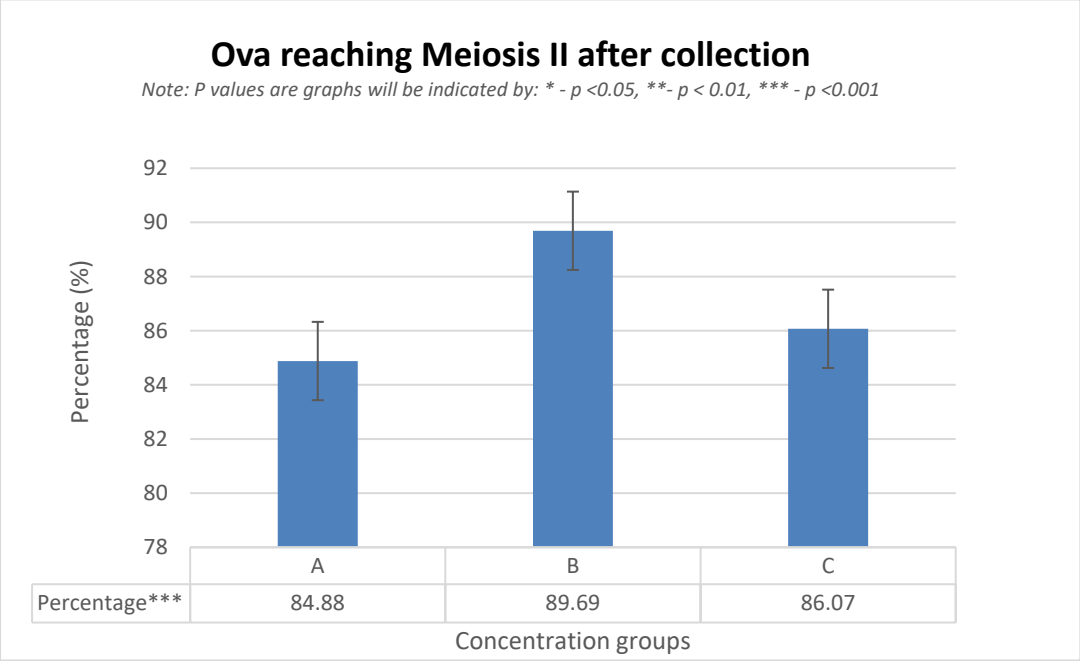


Figure 9: There was a significant difference in the percentage of oocytes which reached the metaphase II stage of development between the three groups ($p<0.001$). Ranges Group A: 82.1 – 86.6, Group B: 87.8 – 91.8, Group C: 87.8 – 90.5.

4.3 Quantity of ova reaching fertilization stage

Post injection all eggs were monitored for the presence of 2 pronuclei in the nucleus, once this was present, the eggs were considered fertilised. There was a significant difference between the three groups in the number of ova achieving fertilization ($p<0.001$). Group A achieving a lower rate of fertilization (68%) compared to groups B and C (>70%). As expected, the percentage of ovum not fertilizing was increased in those with a lower sperm concentration (figure 10)

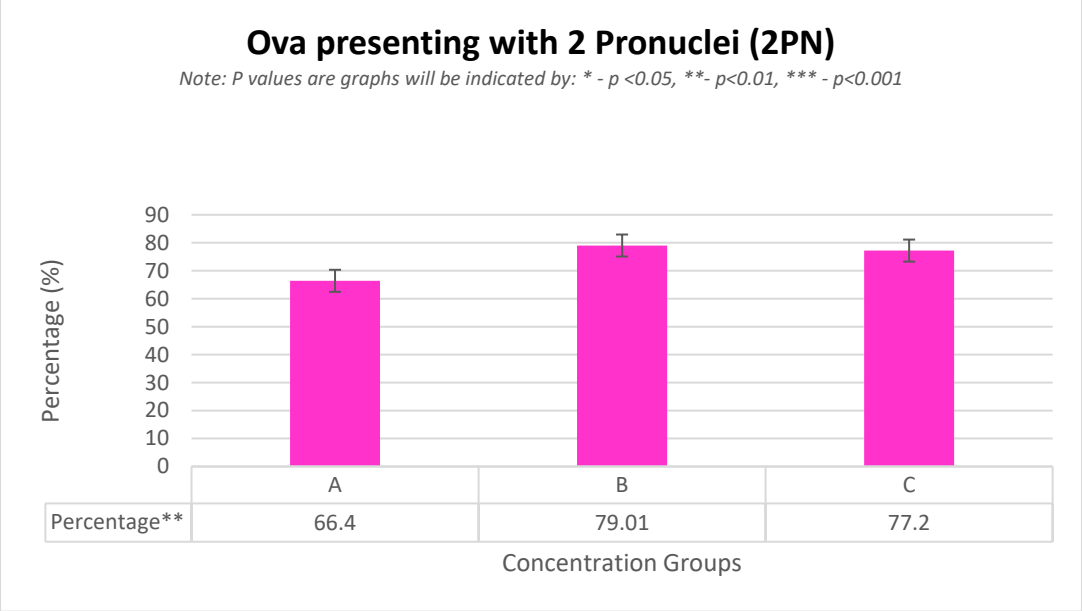
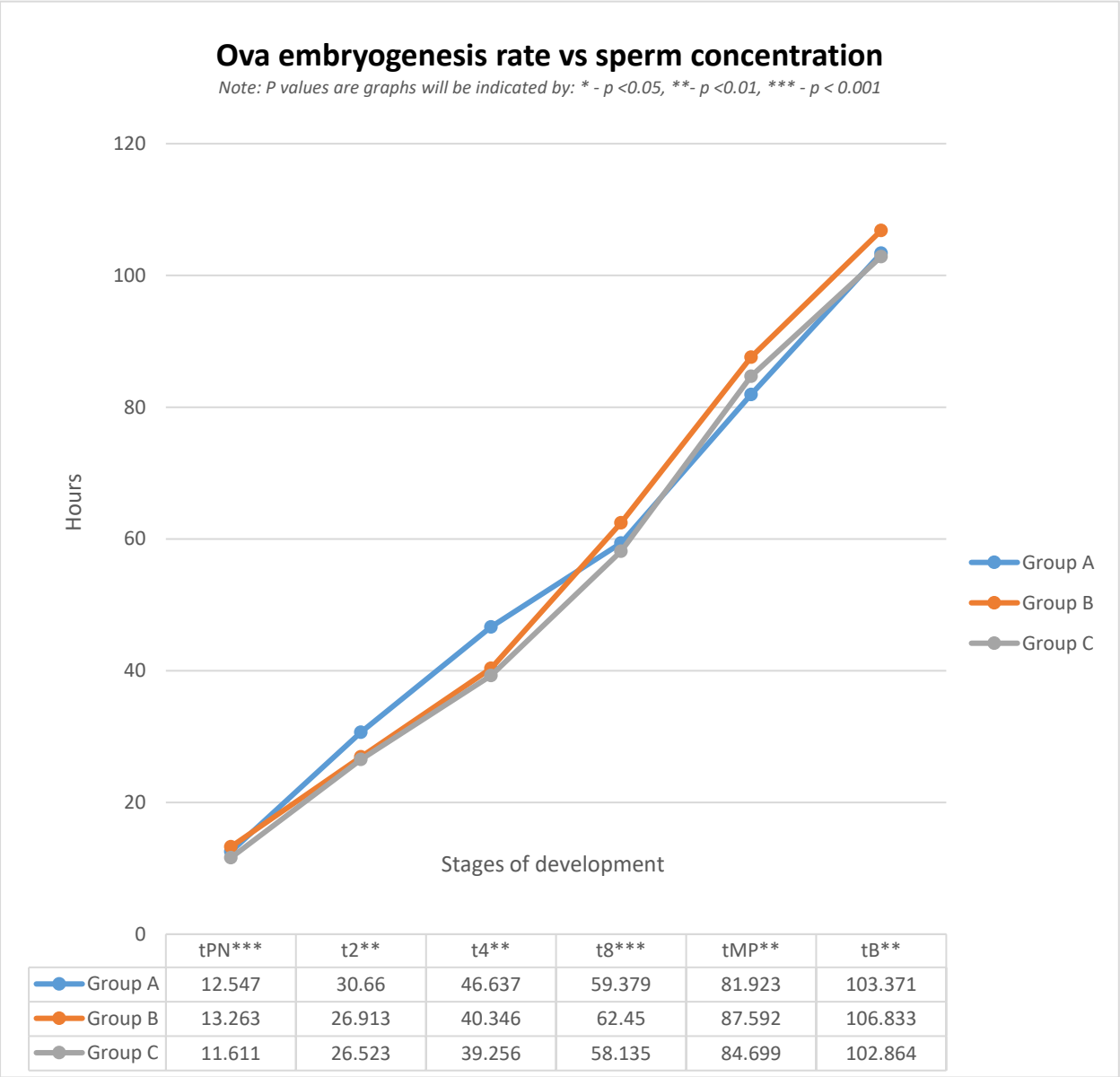


Figure 10: Ova reaching tPN after injection by ICSI procedure. Ranges: Group A: 63.4 – 69.4, Group B: 76.5 – 81.5, Group C: 75.5 – 78.9.

4.4 Sperm concentration and embryogenesis rates

The average rate at which groups A, B and C have progressed through each of the milestone five stages of embryogenesis was similar. However, all five milestones detected by annotation were found to have significant differences between the rate each concentration group reached a growth stage (See figure 11 for graphical representation and table 2 for mean and ranges) (P values: tPN p<0.001, t2 p<0.01, t4 p<0.01, t8 p<0.001, tMP p<0.01 and tB p<0.01).



	tPN***	t2**	t4**	t8***	tMP**	tB**
Group A	12.547	30.66	46.637	59.379	81.923	103.371
Group B	13.263	26.913	40.346	62.45	87.592	106.833
Group C	11.611	26.523	39.256	58.135	84.699	102.864

Figure 11: This graph shows the average rate at which groups A, B and C have progressed through each of the five stages of embryogenesis. All five stages were found to have significant differences between the rate each concentration group reached a growth stage.

Table 3: Fertilization rates

Mean and Ranges of rates of embryogenesis for each of the three concentration groups

Stages of Growth	Sperm Concentration Groups					
	A (<10 million/ml)		B (10 – 50 million/ml)		C (> 50 million/ml)	
	Mean (Range)	SD error	Mean (Range)	SD error	Mean (Range)	SD error
tPN *p<0.001*	12.547 (10.778-14.316)	0.903	13.263 (12.515-14.012)	.382	11.611 (11.116-12.106)	.253
t2 *p<0.01*	30.666 (27.341-33.990)	1.696	26.913 (26.034-27.792)	.448	26.523 (25.463-28.245)	.541
t4 *p<0.01*	46.637 (37.594-55.681)	4.614	40.346 (39.120-41.571)	.625	39.256 (38.085-40.426)	.597
t8 *p<0.001*	59.379 (57.261-61.497)	1.081	62.450 (60.358-64.543)	1.068	58.135 (56.846-59.424)	.658
tMp *p<0.01*	81.923 (78.855-84.990)	1.565	87.592 (84.751-90.432)	1.449	84.699 (79.551-89.847)	2.627
tB *p<0.01*	103.371 (100.088-106.653)	1.675	106.833 (103.339-110.327)	1.782	108.864 (101.285-104.443)	0.806

4.5 Sperm concentration and ova stage progression through embryogenesis

4.5.1 Comparison of ova present at each stage of embryogenesis

After fertilization, the ova were monitored through the five stages of embryogenesis (figure 12 quantitative representation and figure 13 percentage representation). No significance between the groups at each stage of development were noted. However, a statistical downward trend was seen with a significant number of dwindling ova noted for group A ($p<0.01$) and C ($p<0.001$) but not for Group B

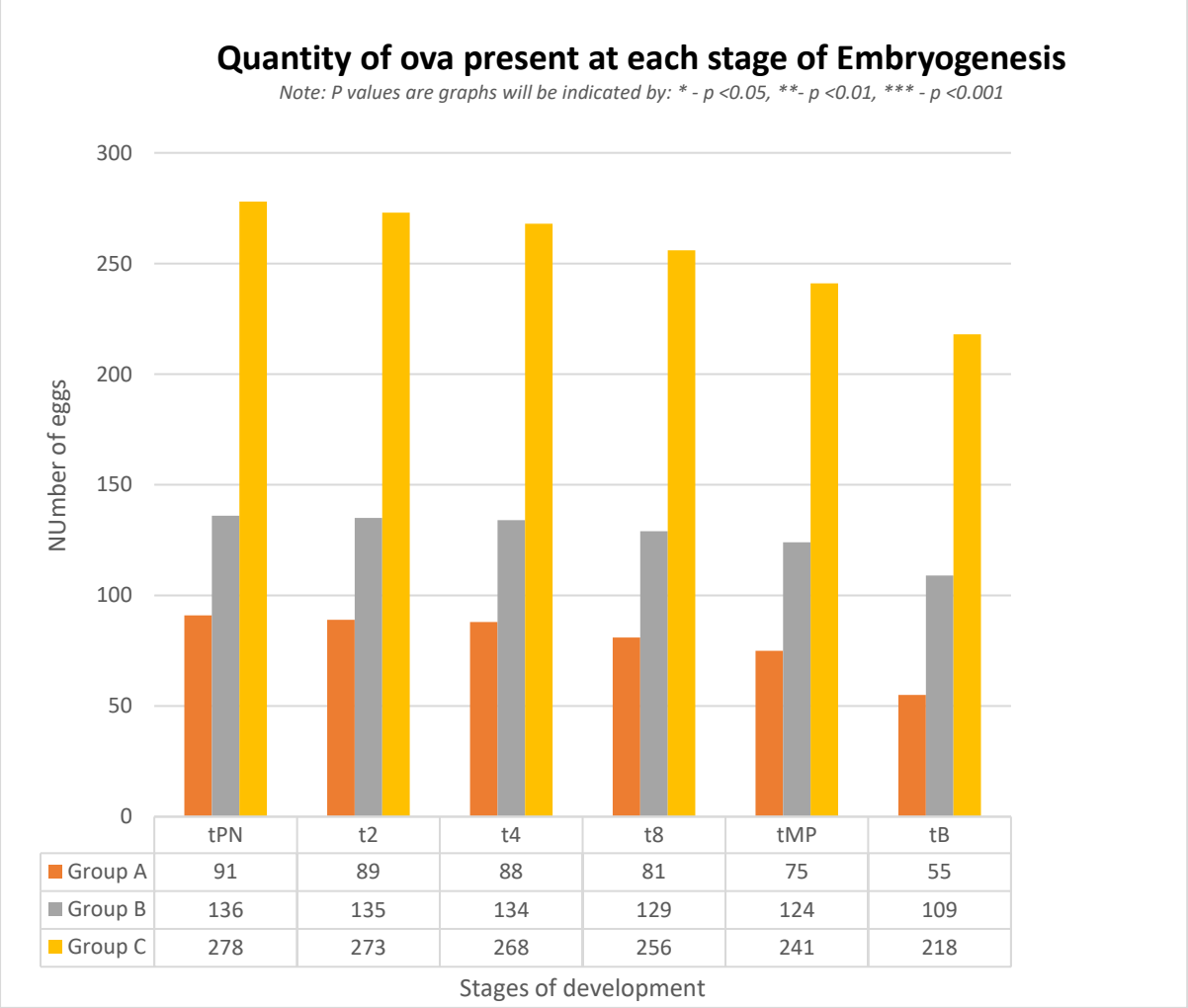


Figure 12: This bar graph shows a quantitative difference in the amount of eggs per group during the five different stages of development.

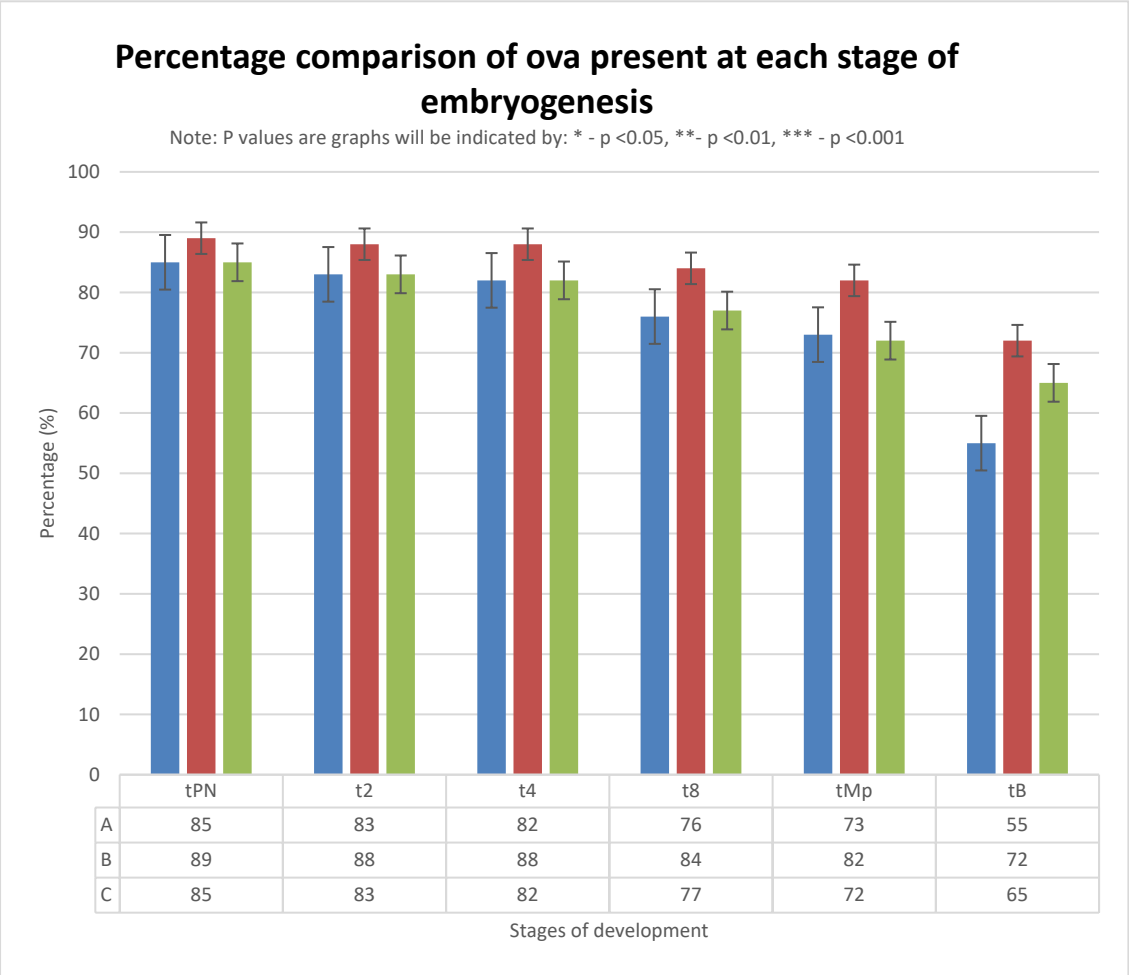


Figure 13: This bar graph shows a quantitative difference in percentage the amount of eggs per group during the five different stages of development.

4.5.2 Identifying at which point in embryogenesis growth is halted

To better identify at which stage of embryogenesis ova growth is halted and to investigate if this differed between the three groups, the five stages of development were broken down into two segments: tPN to t2 (0 - 2) , t4 to tMP (3 - 5) and tB (6). The results of this analysis showed that groups A and B had the most ova not reach milestone stage 3 to 5 while group C in the earlier stage. Those with a low sperm concentration (group A) had a significantly higher loss than either of the other groups ($p<0.01$) (figure 14)

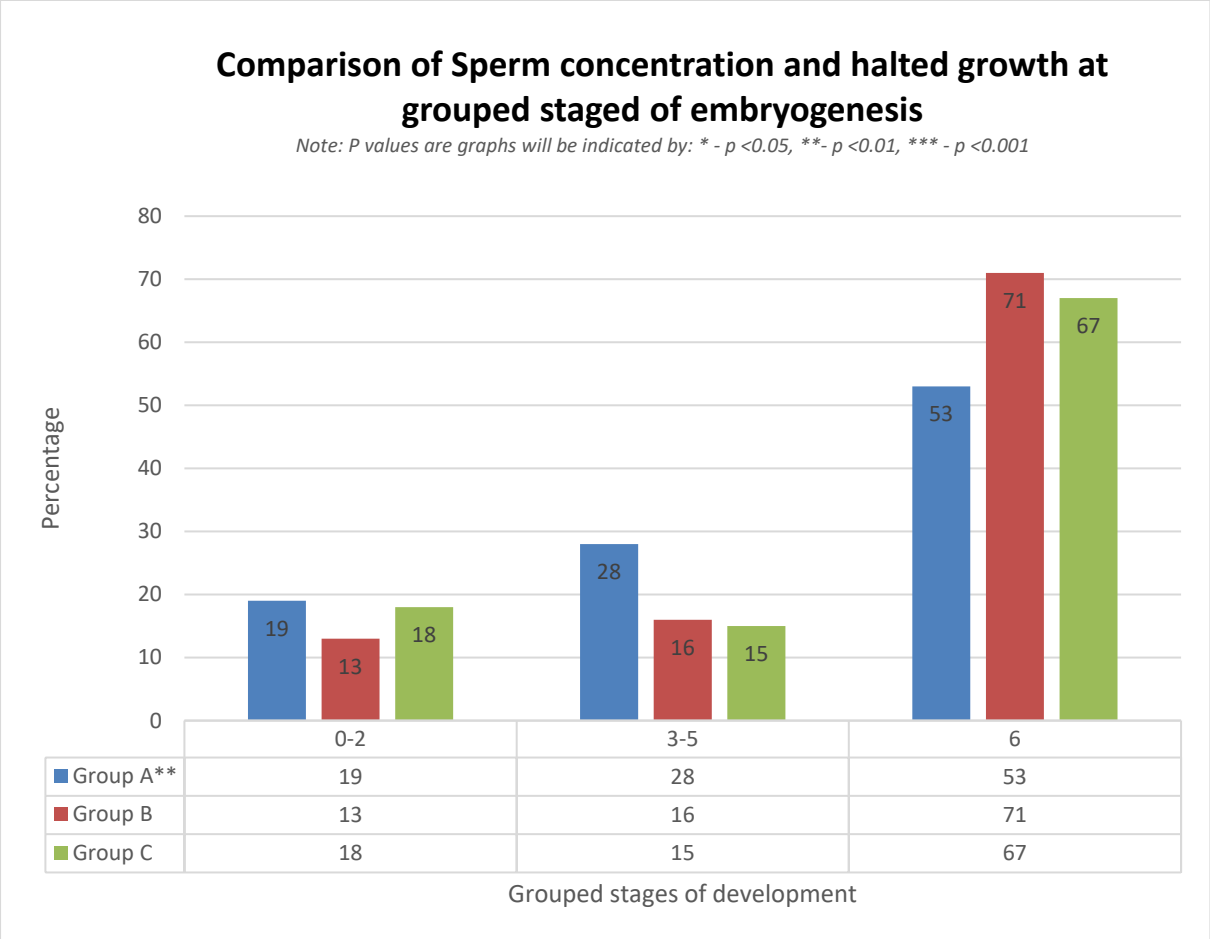


Figure 14: This figure compares the percentage difference between the three groups during the three grouped stages of development.

4.6. Effects of abnormal morphology and motility on fertilisation rates and embryonic development.

The abnormal morphology and motility were analysed as co-variants to concentration and were both identified to have no significant association on the impact of concentration in terms of egg survival as well as rate of growth (Table 4 and 5).

Table 4: Effect of abnormal morphology on sperm concentration:

Concentration	P value significance when compared to other two groups
Group A	Too few samples where morphology was detected
Group B	0.294
Group C	0.801

Too few data points were noted for morphology of Group A as when a semen analysis is performed on a low concentration sample it is done with difficulty as there are too few spermatozoa to examine for an accurate morphology percentage.

Table 5: Effect of motility on sperm concentration:

Concentration	P value significance when compared to other two groups
Group A	1.741
Group B	0.316
Group C	0.223

When motility was made a co-variant on the concentration groups it was noted that motility was not a significant contributing factor to any of the results previously described in point 4.1 – 4.3.

CHAPTER 5

5. Discussion

This study of sixty participants undergoing ICSI at Cape Fertility Clinic has demonstrated that low sperm concentrations of <10million/ml significantly reduce the fertilization rates, increase ovum loss during embryogenesis and have a reduced ability to develop to the blastocyst stage. Covariant analysis revealed that abnormal sperm motility and morphology was not associated with either the impact of concentration or the embryos development.

Figure 9 shows there was a significant difference in the percentage of oocytes which reached the metaphase II stage of development between the three groups ($p<0.001$). Despite this fact there was no significant difference in the overall ovum quality (>80%). This is important as it could affect the overall success rate. The quality of eggs is impacted mainly by the age of a woman, a disorder known as diminished ovarian reserve (DOR) can occur. It occurs when the ovaries are unable to produce high quality eggs over time. It is a common cause of infertility in woman of advanced maternal age (Rasool & Shah, 2017). Low Anti-Mullerian hormone (AMH) levels are used as an indicator for DOR. Younger women can also suffer from diminished ovarian reserve in conditions such as premature ovarian aging (POA) and polycystic ovarian syndrome (PCOS). All women who undergo IVF and ICSI are tested for AMH levels as an indicator for the expected egg quality and volume (De Ziegler et al., 2018).

5.1 Low sperm concentration reduces fertilization rates

Fertilisation is affected by egg quality and sperm quality. In this study we have demonstrated that despite performing ICSI, sperm concentration affected the development of an ovum into a blastocyst. Figure 10 showed there was a significant difference between the three groups in the number of ova achieving fertilization ($p<0.001$). Group A achieving a lower rate of fertilization (68%) compared to groups B and C (>70%). As expected, the percentage of ovum not fertilizing was increased in those with a lower sperm concentration. Predicatively it is said that low sperm counts decrease the chances of pregnancy but does not prohibit pregnancy from occurring (Sharpe., 2012). Spermatozoa affect fertilisation by means of factors such as sperm concentration, sperm motility and deoxyribonucleic acid (DNA) content. In theory ICSI diminishes the effects of motility and concentration as the sperm is injected directly into the ova, however the results of this present study have demonstrated that this is not true. A possible reason for this could be DNA fragmentation.

Sperm DNA fragmentation is a term used to describe the presence of abnormal genetic material within the sperm which may lead to male subfertility. In men, an important factor which may result in sperm DNA fragmentation is oxidative stress. This can be defined as an imbalance between the production of reactive oxygen (free radicals) and antioxidants leading to cell and tissue damage. Oxidative stress can be associated with one or more of the following features: infection, pyrexia, elevated testicular temperature, drug use, smoking, alcohol, stress, pollution, advanced age and diet (Betteridge, 2000).

The reactive oxygen species (ROS) is produced when oxidative phosphorylation within the mitochondria uses nicotinamide adenine dinucleotide (NADH) as an electron donor and oxygen as an electron acceptor in the electron transport chain. This occurs with both reduction and oxidation reaction and the synthesis of adenosine triphosphate (ATP), leading to 1-5% oxygen being transformed into ROS. A second intrinsic source of ROS production in sperm cells is cytoplasmic glucose-6-phosphate dehydrogenase (G-6-PDH). This cytoplasmic ROS has been linked with increased spermatic cytoplasm and infertility. A balance between ROS and antioxidants can keep the spermatozoa at optimum function (Fang & Zhong, 2019).

Low levels of ROS are crucial for fertilization, acrosome reaction, hyper-activation, motility and capacitation. ROS induces cyclic adenosine monophosphate (cAMP) in spermatozoa which leads to the inhibition of tyrosine phosphatase, leading to the phosphorylation of tyrosine. High levels of ROS can promote acrosome reactions with the mechanism of ROS-modulated tyrosine phosphorylation. ROS then generates lipid peroxide radicals which oxidize the lipid rich plasma membrane of the spermatozoa leading to DNA damage (Kemel et al., 2000). IN addition, ROS may directly damage the spermatozoa DNA by breaks in the double or single stranded DNA helix causing chromosomal rearrangements and decreasing the sperm quality. 85% of the spermatozoa's genome is bound in in the central nucleoprotamine, allowing it to be protected from the presence of free radicals. In infertile men, a deficiency in protamination may result in the DNA being susceptible to damage by the ROS (Spiropoulos et al., 2002).

A study by Eversen et al (2002) showed that normal semen samples express a certain level of DNA fragmentation and that oocytes have the ability to correct small amounts of DNA damage upon fertilisation. However, if the damage goes above a certain level it is impossible for the oocyte to cope and fertilization will fail, or embryo development will be impaired. This was further corroborated by Benchaib et al (2003) who established that DNA fragmentation of less than 10% would not negatively affect fertilization and pregnancy rates. However, when

there are large amounts of DNA fragmentation it can cause spermatozoa apoptosis. This was further investigated in a study by Gorczyca et al in 1993 who theorised that intra-testicular programmed death was a way to control clonal expansion of spermatogenesis.

Gorczyca et al (1993) used sperm cells of 25 patients, which were all subjected to analysis of DNA strand breaks using flow cytometry. This was achieved by labelling 3'-OH termini with biotinylated dUTP diphosphatase enzyme in a reaction employing exogenous terminal deoxynucleotidyl transferase. Increased spermatozoa sensitivity to denaturation caused an increase in DNA strand breakage progressing on to programmed cell death and functional elimination from the reproductive pool. Apoptotic death is characterised by the activation of endogenous endonucleases which causes the breakdown of DNA. Apoptotic death is seen in increased amounts in the presence of DNA fragmentation and free radicals. Although sperm concentration is related to the amount of apoptotic death, it is not caused by it and a study by Garcia-Ferreyra (2015) have shown that spermatozoa with DNA fragmentation are still able to fertilise an oocyte but could result in abnormal quality embryo, a block in the blastocyst development and lower pregnancy rates.

However, it has been suggested that in individuals with a lower concentration of sperm, it is more likely that the embryo will be fertilised by a spermatozoon with DNA fragmentation, as the sperm pool is much smaller than that of individuals with normal sperm concentrations (Eygeni et al., 2014). DNA sperm fragmentation can be estimated by using techniques such as the Sperm Chromatin Dispersion (SCD) technique. The principle of this test is that sperm with non-fragmented DNA will produce a characteristic halo of dispersed DNA loops, while those with fragmented DNA fail to achieve this after acid denaturation and the removal of nuclear (McEvoy et al., 2015). Many studies have suggested effects from sperm DNA fragmentation and chromatin packaging to be a great source of decreased fertilisation and cleavage rates and therefore performing a DNA fragmentation test before undergoing ICSI is a valid proposal (Gorczyca et al., 1993; Garcia-Ferreyra, 2015; McEvoy et al., 2015). This test was however not performed in this present study.

5.2 Low sperm concentration has no effect on rate of embryogenesis

Despite the amount of ova not reaching blastocyst stage during embryogenesis, this study has shown that the rate at which each sperm concentration group influenced the embryonic development were similar, with surviving ova reaching the blastocyst stage in <110 hours. Figure 11 showed however that all five stages were found to have significant differences

between the rate each concentration group reached a growth stage (P values: tPN $p<0.001$, t2 $p<0.01$, t4 $p<0.01$, t8 $p<0.001$, tMP $p<0.01$ and tB $p<0.01$). Specific means and ranges for each stage of development monitored can be seen in table 2.

Embryogenesis is the ova's completion of 2 cell divisions up until becoming a blastocyst. The rate at which the ovum goes through the stages of embryogenesis is non-specific. The initial cleavage should occur within the first 12-24 hours, the morula should form within 2-3 days (+/- 72 hours) and the blastocyst within 4 -5 days (+/- 120 hours) (Alpha Scientist in Reproductive Medicine and ESHRE special interest group of Embryology, 2011). Embryo cleavage was assessed using the guidelines for optimal cleavage rate established at the Istanbul consensus workshop in 2011. The schedule is as follows: Day 1 (26 ± 1 h post-ICSI, 28 ± 1 h post-IVF), 2-cells; Day 2 (44 ± 1 h), 4-cells and Day 3 (68 ± 1 h), 8-cells (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). The occurrence of an early cleavage before 26±1 h (ICSI) and 28±1 h (IVF), has been shown to be associated with good quality embryos, blastocyst development and pregnancy rates (Lundin et al., 2001; Fenwick et al., 2002).

Research has been done to show that concentration groups have no effect on rate of embryogenesis. A study of 136 couples who were divided into two groups, one with abnormal sperm concentration and the other with normal sperm concentrations, were monitored for rate of embryogenesis after insemination and no significant difference was observed (Gao; 2006). Many other studies have further noted that cleavage rate should not be monitored but rather the presence of the cleavage itself. It has thus been well established that the synchronicity of division is of greater impact than the rate at which the division occurs (Scott et al., 2008; Lemmen et al., 2008; Wong et al., 2010; Meseguer et al., 2011; reviewed by Kirkgaard et al., 2012). The results of our study have confirmed these studies although those with a low sperm count had an increase in ovum loss, the rate of embryogenesis was similar between the three groups.

5.3 Low sperm concentration and its effect on embryogenesis

After fertilization, embryogenesis was monitored and while all concentration groups had a steady decline of viable eggs those with low sperm concentrations had less embryos reaching the blastocyst stage. Figure 12 and 13 showed there was a statistical downward trend present with a significant number of dwindling ova noted for group A ($p<0.01$) and C ($p<0.001$) but not for Group B. To better identify the stage of embryo development ovum growth is affected and

to investigate if this differed between the three groups. The results seen in figure 14 of this analysis showed that groups A and B had the most failed ovum in the later stages while group C in the earlier stage. Those with a low sperm concentration (group A) had a significantly higher failure rate than either of the other groups ($p<0.01$)

In normal pregnancy embryogenesis begins with the first cytokinesis whereby the embryo splits itself into two blastomeres of equal size (Magli et al, 2012; Garcia-Ferreyra, 2015). The two blastomeres then divides, giving rise to four blastomeres of the same size. The divisions are not synchronous, resulting in transient brief periods of odd numbers of blastomeres. The blastomeres continue to divide and multiply until they form the central mass found in a blastocyst. When abnormal cytokinesis occurs, the division is haltered causing embryogenesis to stop (Gardner & Balaban, 2016; Mahagan et al., 2018).

There are many factors that cause the halting of embryogenesis such as: paternal genetic factors, growth environment and egg quality. The male provides half of the genetic material to an embryo but also contributes the centrosome that helps the embryo to differentiate. The sperm further contributes to activation factors which stimulates the zygote to complete its first cellular division (Burruel et al., 2014). As the sperm fuses with the oocyte membrane it releases a sperm specific phosphatase C-zeta with the addition of intracellular calcium oscillations which triggers the oocyte activation factor enabling the first cellular division (Yeste et al., 2017).

In normal pregnancy, the growth environment would be *in utero* at a site along the fallopian tube. Fluid produced and secreted by the fallopian tube provides the environment in which gametes are transported to the uterus in which oocytes are fertilized and early embryo development occurs. It is composed of oviductal fluid in terms of ions and nutrients such as glucose, lactate, pyruvate, and amino acids (Briceag et al., 2015). During ICSI and IVF, culture media is used to create a stable environment similar to the fallopian tube. With the addition of the incubation environment of controlled oxygen, temperature, and pH the Embryoscope should act as the prime environment for the growth of the embryo (Ng et al., 2018).

A retrospective study has shown that the combination of the Embryoscope with a morpho-kinetics model resulted in a relative increase in clinical pregnancy by 20% when compared with culture in a standard incubator (Meseguer et al., 2012). This study was challenged by a large randomised controlled trial to determine whether incubation in the integrated

Embryoscope time lapse monitoring system (TMS) and selection supported by the use of multivariable morpho kinetic models improve reproductive outcome in comparison with a standard incubator. The embryos from 843 infertile couples undergoing ICSI were monitored. This trial reported an overall implantation rate increase of 21% and an ongoing pregnancy rate increase of 23.2% when the Embryoscope was compared to standard incubation (Rubio et al., 2014). This advantage of the Embryoscope was again demonstrated more recently in a study which noted that the use of the Embryoscope increased the number of blastocysts formed and was probably due to a suitable culture environment which does not affect the embryo quality at cleavage stage and blastocyst development. (Sciorio et al., 2018).

5.4 Motility and Morphology have no significant impact on embryo development or fertilization

Abnormal morphology and motility was assessed as a co-variant for each couple in this study and was shown to have no effect on the outcome of the rate of growth, fertilization or development of the embryos, see tables 4 and 5. This has been supported by the results reported by Kellerman and Lombard, 2007 who studied the effects of sperm morphology on pregnancy outcomes and noted that abnormally classified sperm did not interfere with fertilising capacity. Normal spermatozoa constitute only >5% or more of the entire sperm concentration which is relatively non-specific. This study monitored both IVF and ICSI (Kellerman & Lombard, 2007).

Normally sperm would be required to penetrate the zona pellucida to fertilise the egg. If the motility is low (normal range: >32%) this could negatively impact all eggs for fertilisation. The importance of high sperm motility was investigated in 41 patients undergoing IVF and it was shown that those with low motility only had a 14.6% pregnancy rate (Chye et al., 1986). In this present study all patients underwent ICSI which has been shown to not be influenced by the motility of the sperm as the sperm is directly injected into the egg and therefore eliminates the need for the sperm to swim effectively in order to penetrate the ova.

A relationship between sperm morphological abnormalities and embryonic morphokinetic parameters was seen in a study by Nikolova *et al*, 2020 the research highlighted a significant correlation between sperm defects of the head, midpiece and tail with stages of embryonic development from observation of both pronuclei to the hatched blastocyst. With certain sperm defects such as a coiled tail having a negative impact on embryo morphokinetics and

implantation success (Nikolova *et al*., 2020). This is the opposite result of the findings in this study, this may be due to a fewer number of sperm samples checked for abnormal morphology. A larger study population would be required to better analyse the true impact of sperm morphology on embryogenesis and embryo morphokinetics.

5.5 Sperm concentration has no significant impact on successful positive BHCG post embryo transfer

The results in table 2 show that group A and C were seen to have positive BHCG results in more than 40% of the cases after embryo transfer whilst group B only had a positive BHCG value in less than 25% of the cases in this study. The results were seen to be insignificant. In a study by Wiesak *et al*, it was seen that with increased sperm concentration there was a decrease in clinical pregnancy outcomes (p,0.01) and implantation (p<0.02). The results in this study are however do follow the same pattern with Group A and B but not C, more data would be required to better confirm the same outcome (Wiesak *et al*, 2019).

5.6 Limitations of this study

A major limitation of this study was the low number of participants in each group which made statistical comparison difficult in respect to motility and abnormal morphology. A further limitation was that tests such as DNA fragmentation was not performed. The DNA fragmentation measured could provide essential information and a possible explanation for the results obtained in this study. Follow up studies could also be done to see the success of the pregnancy in comparison to the results in this study.

5.7 Advantages of this study

Despite the above limitations, the study has been able to demonstrate that low sperm concentration does influence the capability of embryos to reach the blastocyst stage of development. The results indicate that sperm concentrations of <10million/ml have a lower chance of reaching the blastocyst stage by negatively affecting different embryonic milestones. It also shows that males with a sperm concentration of <10million/ml have a lower chance of fertilizing eggs as seen in the results previously stated. The methodology and analyses used in this investigation were all performed according to strict WHO guidelines

(World Health Organisation Department of Reproductive Health and Research; 2010) and were completed in a highly controlled laboratory environment.

CHAPTER 6

6. Conclusion

The findings of this study have demonstrated that male participants with low sperm counts had significantly lower fertilization rates and reduced percentages of embryos reaching the blastocyst stage of development. This indicates that males with sperm concentration <10 million/ml have a less likely chance of being able to fertilize an egg and if fertilization occurs a less likely chance of the ovum reaching blastocyst stage in order for the ova to implant. There was however no statistical correlation between the rate of growth, this indicates that sperm concentration does not impact how the fertilized ova go through embryogenesis. Abnormal morphology and motility had no impact on the results due to a lack of participant data further iinvestigations and increased data would be required to understand the impact if any these parameters may have on embryogenesis and decreased sperm concentrations.

This is an important finding however further investigation on a larger number of participants would be required to confirm these results. Additional investigations to further explain the mechanisms of these findings should be performed. These should include DNA fragmentation studies and the impact of oxidative stress on both semen quality and concentration. Other further investigations to made should be identifying if male age is an increasing factor on sperm concentrations. The effects of female factors on embryogenesis such as hormones like follicle stimulating hormone and luteinizing hormone levels can also be investigated.

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Appendix A

CAPE FERTILITY
We value each individual patient

DRS HEYLEN LE ROUX AND MATEBESE INC
Reproductive Medicine Specialists Pr 0152358
Reg. No. 2000/022451/21 • VAT No. 4740196128
Suite 209 Library Square, 1 Wilderness Road, Claremont 7708
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DR SULAIMAN HEYLEN MD (KUL), Obs&Gyn (KUL), Reprod Med.
DR PAUL LE ROUX MBChB (UCT), FCOG (SA), Reprod Med.
DR NOMATHAMSANQA MATEBESE MBChB (NATAL), FCOG (SA), MMed (UCT), Reprod Med.
DR LIZLE OOSTHUIZEN MBChB (UCT), FCOG (SA), MMed (UCT)

25 January 2017

Re: Thesis Prospectus for Masters of Science in Medical Laboratory Sciences:

Thesis topic: the effect of varying decreased sperm concentrations on embryogenesis by the use of EmbryoScope time lapse monitoring.

Rizqa Siebritz

Completion of degree: Masters of Sciences in biomedical technology

CPUT supervisor: Glenda Davison.

Cape Fertility Clinic Supervisor: Dr Sulaiman Heylen and Dr Paul le Roux

Ms. Rizqa Siebritz has been in contact with the Cape Fertility Clinic regarding the use of the EmbryoScope for her master's project. In this study she will study embryo development with time-lapse technology of couples where the males have decreased sperm counts or sperm dysfunction and determine if it is an affecting factor. We are happy to accommodate this request and will provide with the necessary support.

We will need to have ethics approval of CPUT, without ethics approval we will not be able to proceed.

The project will be purely review of data. The treatment and the outcome for the patients will not be influenced by this project. And therefore a patient consent is not required.

Rizqa will need to sign a confidentiality agreement. Which will be designed once ethical approval has been obtained.

Please let us know if you require more information.

Dr Sulaiman Heylen
Obstetrician and Gynecologist
Subspecialist in Reproductive Medicine

Dr Paul le Roux
Obstetrician and Gynecologist
Subspecialist in Reproductive Medicine

APPENDIX B

CAPE FERTILITY
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DR SULAIMAN HEYLEN
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DR NOMATHAMSANGA MATEBESE
MBChB (HASK), FCOG (SA), MMed (UCT), Reprod Med.

DR UZLE OOSTHUIZEN
MBChB (UCT), FCOG (SA), MMed (UCT).

CONSENT TO TREATMENT INVOLVING IVF (IN-VITRO FERTILIZATION) / ICSI (INTRA CYTOPLASMIC SPERM INJECTION) AND EMBRYO TRANSFER

We.....
(Full name of husband / male partner)

AND

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

OF (address)

.....

.....

.....

Hereby state:

1. I consent to hormones and other drugs being administered to me to stimulate the development of follicles which contain the eggs in my ovaries.

2. I consent to egg retrieval by means of transvaginal ultrasound under anaesthesia / conscious sedation.

3. I understand that if less than three pre-ovulatory follicles develop in my ovaries in response to stimulation my egg collection procedure may be cancelled.

4. 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 at a later specified date.

5. The sperm to be used to endeavor to make the wife/female partner pregnant will be that of:

   ☐ the husband/male partner

   ☐ an anonymous sperm donor. Code .....

   ☐ a known sperm donor. Name .....

6. Cycle cancellation. We understand that failed fertilization of eggs and failure to culture embryos are possible complications 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 Cape Fertility 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.

7. I understand that although the risks of IVF are rare, they include the following:

   - **Ovarian hyperstimulation syndrome (OHSS)**. Severe OHSS (1% of cases) may result in swelling of the ovaries, fluid accumulation in the abdomen and lungs, organ failure and vascular thrombosis.

   I understand that I should contact the fertility clinic urgently if I have any symptoms of OHSS (severe abdominal pain, nausea and vomiting, dizziness, shortness of breath or decreased urine output) for assessment by one of the doctors in the clinic. I understand I may need hospital admission if I develop OHSS.

   - **Pelvic infection.** This can result in damage to my fallopian tubes, ovaries and uterus. Severe pelvic infection can result in removal of my tubes, ovaries or uterus (hysterectomy). Severe infection can also result in general organ failure.

   - **Ovarian torsion** (twisting of an ovary), which may necessitate surgical removal of the ovary.

   - **Anesthetic complications.** I understand that I will have my egg collection under anaesthesia, and accept the associated risks. I understand that a specialist

anesthesiologist will be present to perform my procedure.

8. The doctor has explained to us the nature and implications of the procedure and we understand that there is no guarantee of success. We understand that even though the IVF treatment 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.

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

10. We understand that if pregnancy results there is the possibility, as with any pregnancy, of complications of childbirth or 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 alerted prevalence of preeclampsia. In men with a very low sperm count, utilizing ICSI (Intra Cytoplasmic Sperm Injection) treatment, there may be an increased prevalence of low sperm count in male offspring.

11. I consent to the photographing or televising of any laboratory procedure(s) to be performed for medical, scientific, or educational purposes, provided my identity is not revealed by the pictures or by descriptive text accompanying them. I understand that all information about myself obtained during the program will be handled confidentially and that my identity or specific medical details will not be revealed without my written consent. Specific medical details may be revealed in professional publications as long as my identity is concealed.

SIGNED AT.....

THIS.....DAY OF.....2017

AS WITNESS.....

HUSBAND/MALE PARTNER.....

WIFE/FEMALE PARTNER.....

Cape Peninsula University of Technology

APPENDIX C

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: sethn@cput.ac.za

12 March 2018
REC Approval Reference No:
CPUT/HW-REC 2018/H3

Dear Ms Rizqa Siebritz

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to Ms Siebritz for ethical clearance. This approval is for research activities related to student research in the Department of Biomedical Technology at this Institution.

TITLE: **The effect of varying decreased sperm concentrations on embryogenesis by the use of embryoscope time lapse monitoring**

Supervisor: Prof G Davison and Dr M Heylen

Comment:

Approval will not extend beyond 13 March 2019. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Mr. Navindhra Naidoo
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences