Evaluating the Role of Hyaluronic Acid Products In the Treatment of Infertility

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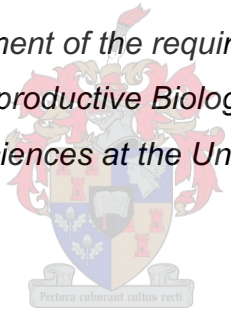

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Declaration

By submitting this thesis, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2017

Abstract

Hyaluronic acid is (HA) a naturally existing macromolecule and present in several HA based products aiming to improve the outcomes of assisted reproduction. Hyaluronic acid is produced by the human cumulus cells and is therefore naturally present in the female reproductive tract. During physiological intracytoplasmic sperm injection (PICSI®), spermatozoa that are able to bind to solid-state HA, have a chromatin structure with high DNA chain integrity associated with fertilization competence and normal chromosomal constitution. The addition of HA into transfer media is based on several properties that characterize it as an implantation-enhancing molecule. Its viscosity is believed to facilitate the integration of the embryos into the fluid secretions of the intrauterine environment as it has been shown to increase cell-to-cell and cell-to-matrix adhesion. The presence of the CD44 receptor for HA, expressed on both the embryo and the supporting network of the human endometrium, suggests the action of HA during the process of implantation.

The aim of the study was to evaluate retrospectively the role of hyaluronic acid in the PICSI® procedure and prospectively also its use in embryo transfer medium.

The retrospective study involved the review of 388 patients whose treatment involved the fertilization of their ova by either physiological intracytoplasmic sperm injection (PICSI®) or traditional intracytoplasmic sperm injection (ICSI). *In vitro* fertilization (IVF) was used as a control group. PICSI® involves the use of a specialised culture dish that has three gel-like microdots of HA attached to the bottom surface of its interior. These HA dots provide an additional parameter whereby spermatozoa with a normal chromosome composition can be selected for injection. Strict exclusion criteria were applied to eliminate variables other than the fertilization method that could have influenced treatment outcomes. The comparison showed no statistically significant improvement in ART outcome, at Drs Aevitas Fertility Clinic (Vincent Pallotti Hospital, Pinelands, South Africa), when PICSI® dishes were used to select spermatozoa for injection. Outcomes measured included; fertilization rate, embryo quality, pregnancy and miscarriage rates.

The prospective study evaluated the treatment outcomes of patients who underwent embryo transfer with specialized embryo transfer medium, EmbryoGlue® (EG) in comparison with a historically similar patient population who underwent embryo transfer with traditionally employed culture media at the same clinic (Drs Aevitas Fertility Clinic). The HA contained in EG acts as a specialised adherence compound supporting the implantation of a transferred embryo in the endometrium. The comparison showed no statistically significant improvement in clinical pregnancy, ongoing clinical pregnancy nor in miscarriage rates when adjustments were made for confounding factors.

The results of the study have produced valuable information that will inform future use of HA products in the treatment of patients attending Drs Aevitas Fertility Clinic.

Opsomming

Die makromolekule, hualuroonsuur (HS), wat natuurlik voorkom, is teenwoordig in verskeie produkte wat die uitkoms van geassisteerde reproduktiewe tegnieke (GAT) kan verbeter. Hualuroonsuur word deur menslike kumulusselle geproduseer en is dus natuurlik teenwoordig in die vroulike reproduktiewe traktus. Tydens fisiologiese intrasitoplasmatiese sperm inspuiting (PICSI®), bind spermatosoa met hoë DNA integriteit, wat geassossieer word met bevrugtingspotensiaal en normale chromosomale samestelling, aan die hualoroonsuur in gelvorm. Die byvoeging van hualoroonsuur in embrio terugplasingsmedia word gebasseer op die eienskap dat die viskositeit van hualoroonsuur die integrasie van die embrio met die sekresies van die intra-uteriene holte en dus ook inplantasie bevorder. Dit word toegeskryf aan beter sel tot sel en sel tot matriks adhesie. Die teenwoordigheid van CD44 reseptore vir HA, teenwoordig op beide die embrio en die endometrium, bevestig die rol van hualoroonsuur tydens die inplantasie proses.

Die doel van die studie was om die rol van hualoroonsuur tydens die PICSI® prosedure sowel as die embrio terugplasingsprosedure te bepaal.

Die restrospektiewe studie, wat 388 pasiente ingesluit het, het gepoog om die produk PICSI® wat hualoroonsuur bevat, te vergelyk met klasieke intrasitoplasmatiese sperm inspuiting [ICSI]. Die kontrole groep was pasiente wat met behulp van die in vitro bevrugtings metode [IVB] behandel is. Tydens die PICSI® prodsedure word ‘n gespesialiseerde kultuur bakkie, wat drie druppels HS in gelvorm op die oppervlak bevat, gebruik. Hierdie hualoroondruppels bied die voordeel dat spermselle met normale chromosoomsamestelling geselekteer kan word. Streng uitsluitingskriteria is toegepas om alle ander veranderlikes wat uitkoms kan beïnvloed, te elimineer. Die vergelyking van data by die Drs Aevitas fertiliteitskliniek (Vincent Pallotti Hospitaal, Pinelands, Suid Afrika),het geen statisties beduidende verbetering in uitkoms tydens die PICSI® prosedure aangedui nie. Uitkoms is gemeet aan persentasie bevrugting, embriokwaliteit, swangerskap- en miskraam koers.

Die prospektiewe studie het die behandelingsuitkoms tussen pasiente wat embrioterugplasing ondergaan het met EmbryoGlue® (EG) medium en histories soortgelyke pasiente wat histories embrioterugplasings ondergaan het met tradisionele kultuur medium, by dieselfde kliniek (Drs Aevitas fertiliteitskliniek) vergelyk. Die EG medium bevat hualoroonsuur wat volgens studies implantasie van die embrios bevorder. Die resultate het, na aanpassing van strengelveranderlike faktore, geen stasties beduidende verbetering in kliniese swangerskap, voortgaande swangerskap of miskraam koers tydens die gebruik van die EG medium aangedui nie.

Die resultate van die studie het waardevolle inligting rakende toekomstige gebruik van HS produkte tydens die behandeling van pasiente by die Drs Aevitas fertiliteitskliniek verskaf.

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This work has been presented in Video Conference at Drs Aevitas Fertility Clinic as well as the University of Stellenbosch Academic Year day 2016, in the form of a poster presentation.

Dedication

Dedicated to my grandmother, Theunsina Maree,

as a token of my admiration and affection.

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Abbreviations

a-CGH	Array Comparative Genomic Hybridization
ART	Assisted reproductive techniques
ASRM	American Society of Reproductive Medicine
CBAVD	Congenital bilateral absence of the vas deferens
CGH	Comparative genomic hybridization (CGH) or whole genome amplification (WGA)
CP	Clinical pregnancy
CPR	Clinical pregnancy rate
COC	Cumulus-oocyte complex
CVS	Chorionic villus sampling
DNA	Deoxyribonucleic acid
EG	EmbryoGlue®
ESHRE	European Society of Reproduction and Gynaecological Endoscopy
FSH	Follicle stimulating hormone
GnRHa	Gonadotropin-releasing hormone agonist
GQE	Good quality embryos
HA	Hyaluronic acid / Hyaluronan
HBA	Hyaluronan binding
HCG	Human chorionic gonadotropin
HMG	Human menopausal gonadotropin
ICMART	International committee for monitoring assisted reproductive technology
ICSI	Intracytoplasmic sperm injection
IMSI	Intracytoplasmic morphologically selected sperm injection
IR	Implantation rate
IUI	Intrauterine insemination
IVF	*In vitro* fertilization
LBR	Live birth rate

MACS	Magnetic activated cell sorting
MESA	Microsurgical epididymal sperm aspiration
MSOME	Motile sperm organellar morphology examination
OAT	Oligoasthenoteratozoospermia
OP	Ongoing pregnancy
OPR	Ongoing pregnancy rate
OD	Odds difference
OHSS	Ovarian hyperstimulation syndrome
PCOS	Polycystic- ovarian syndrome
PESA	Percutaneous sperm aspiration
PGS	Preimplantation genetic screening
PICSI®	Physiological intracytoplasmic sperm injection
PVP	Polyvinylpyrrolidone
PVS	Perivitelline space
PZD	Partial Zona Dissection
RCT	Randomized controlled trials
SARA	South African Register of Assisted Reproductive Techniques
SUZI	Subzonal insemination
TESE	Testicular sperm extraction
TLM	Time-lapse kinetic monitoring
WGA	Whole genome amplification
ZP	Zona pellucida

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Chapter 1

Background Information & Literature Review

Introduction

Assisted reproductive techniques (ART) treatments involve the application of sophisticated ovarian stimulation protocols, continuous patient monitoring and delicate follicle aspiration whereby mature oocytes are harvested and fertilized in the *in vitro* laboratory setting. The embryos are then cultured in specialized media for approximately 5 days before a variety of invasive and non-invasive techniques may be employed to select, what we believe to be, the most implantation competent embryos for transfer. A result of failed implantation often baffles clinicians and scientists alike and necessitates further investigation into ways in which we can support embryo implantation; improve clinical pregnancy and live birth rates (Balaban, *et al*, 2011). Routine practice also involves a semen analysis for the male partner before assessing the female partner. If abnormal parameters are encountered during routine semen analysis, the addition of functional sperm tests may be used to further assess sperm normalcy and fertilization capabilities (Jeyendran *et al*, 1984; World Health Organization, 2010).

Continuous research to improve ART outcome also involves amongst others, improved sperm selection methods (Appendix VII & VIII) in advanced *in vitro* fertilization methods and improved adhesion and implantation to the endometrium (Achache *et al*, 2006). In the current study the effect of physiological intracytoplasmic sperm injection (PICSI®), a sperm selection method and the use of EmbryoGlue® (EG) transfer medium, a hyaluronic acid (HA) based product, on ART outcome will be specifically investigated.

1.1 The History of Assisted Human Reproduction

1.1.1 The History of IVF

Our collective understanding of the molecular mechanisms involved in the fertilization process began with studies in non-mammalian species, such as amphibians, where fertilization occurs outside the body. The first reported observation of sperm penetrating an oocyte was in 1852 and published the year thereafter (Clarke, 2006). Subsequent non-mammalian studies further helped define the specific experimental conditions that enabled the first successful *in vitro* fertilization of mammalian oocytes in 1959 (Bavister, 2002).

In 2015 a collection of papers published in Reproductive Biomedicine & Society Online (Elder & Johnson, 2015a, Elder & Johnson, 2015b) describes the collaboration of Robert Edwards and Patrick Steptoe in the years spanning 1969-1978 that ultimately lead to the birth of the world's first IVF baby, Louise Joy Brown on 25 July 1978. It should be mentioned however, that the true origins of IVF date back as far as 1878 to basic research studies and work on hamster and rabbit gametes (Yanagimachi & Chang, 1964). The success of Edwards & Steptoe in achieving the world's first IVF birth is therefore also owed to numerous pioneering scientists who came before them. In late 1968, Edwards & Steptoe initially focussed their collective efforts on perfecting all elements of the laparoscopic oocyte retrieval procedure including the technical aspects and instruments necessary, induction of ovulation, timing of aspiration and ovarian stimulation protocols.

Aspiration equipment similar to that used in modern ART clinics was developed in September 1969 (Elder & Johnson, 2015a). A bypass valve allowed the optimization of suction pressure used to aspirate the follicles after it was determined that pressure greater than 12cm Hg may result in oocyte damage (Elder & Johnson, 2015a). The optimal timing of human chorionic gonadotropin (HCG) administration, used to induce the maturation of follicles, was indicated by the presence of urinary oestrogens in excess of 75ug/day detected in 24-hour samples (Elder & Johnson, 2015a). The analysis of recovered research notes from 1969 indicated that HCG was administered between 29-31 hours before aspiration. The timing of HCG trigger was based on availability of the theatre & operating team rather than maturation of the follicles. The dosage of HCG used for trigger ranged between 3000 and 12 000IU and was based on the doses of gonadotropins used for follicular stimulation in the cycle. A standard protocol for follicle stimulation and maturation, consisting of three injections of human menopausal gonadotropin (HMG) between days 2-9 and 5000IU of HCG on days 9-11 of the menstrual cycle was later adopted (Elder & Johnson, 2015a). Edwards & Steptoe used the presence of a corpus luteum

to determine whether ovulation had taken place prior to oocyte retrieval as the duration between HCG administration and the retrieval procedure varied. Oocyte retrieval was carried out on days 10-12 of the menstrual cycle and the interval between HCG trigger and oocyte retrieval was gradually adjusted from 28.75-29.50 hrs to 32.0-33.5 hrs in March 1970 (Elder & Johnson, 2015a).

1.1.2 Follicle Stimulation Regiments

Edwards & Steptoe optimized their ovarian stimulation regiments by adjusting the doses of HMG to regulate follicular development, and HCG to induce follicular maturation, in stimulated patient cycles over a number of years (Elder & Johnson, 2015b). The resulting follicle measurements, number and apparent maturity of oocytes retrieved were compared among different stimulated cycles and their findings published in *Nature* magazine in 1970 (Edwards, Steptoe & Purdy, 1970). The initial doses of HMG administered were conservative considering the lack of available information regarding potential ovarian response as well as the potential risk of patients developing ovarian hyperstimulation syndrome. In 1969 two ampules of Pergonal were administered in a single dose early in the menstrual cycle. The number of HMG ampules administered steadily increased to three doses of two, three or four ampules on days 6, 8 and 10 of the menstrual cycle after it was observed that lower doses were effective only in extending time to menstruation. They succeeded in taking control of patients' menstrual cycles with repeated injections of these higher doses of HMG and by 1972 the success rate in oocyte retrieval had increased from 83% to 95%. In 1973 high doses of HMG were found to result in increased oestrogen production and secretion during the follicular phase of stimulated cycles. Edwards & Steptoe began to replace Pergonal with clomiphene citrate stimulation as high oestrogen levels during the follicular phase had negative consequences on the resulting luteal phase endometrium (Elder & Johnson, 2015b). The use of HCG to induce follicle maturation in natural cycles was also attempted in an effort to address the elevated oestrogen levels. In the period between 1973 and 1978 Pergonal-stimulated, clomiphene citrate-stimulated and natural cycles were carried out in parallel but none resulted in the establishment of an ongoing pregnancy (Elder & Johnson, 2015b). Edwards & Steptoe changed their approach in 1978, abandoning stimulation with exogenous hormones focussing instead on meticulous endocrine monitoring, including urinary total oestrogen in natural cycles. They reported greater success in the retrieval of functionally mature oocytes in natural cycles (Elder & Johnson, 2015b).

In addition to the ground-breaking work of Edwards & Steptoe, the tireless efforts of scientists and clinicians that followed over the last three decades have informed the collective present-day understanding of all aspects of human reproduction. Modern techniques for ovarian

stimulation, oocyte retrieval, assisted fertilization, embryo culture, sperm preparation and cryopreservation have made possible the birth of some 5 million babies worldwide (Mansour *et al,* 2014).

1.1.3 Standard IVF

Standard IVF is a process whereby good quality spermatozoa are added to the immediate culture environment surrounding retrieved mature oocytes and incubated together at 37 ºC (6% CO_2) overnight. The cumulus complex is stripped from each oocyte the following morning to allow fertilization evaluation (Appendix XII Standard Operating Protocol: In Vitro Fertilization). Successful fertilization, indicated by the presence of two pronuclei and two polar bodies, cannot however be achieved through standard IVF when there are severe male factors contributing to the etiology of a couples' infertility. The percentage of normally fertilized oocytes and number of embryos generated by conventional IVF is significantly lower when semen characteristics were below reference ranges in terms of concentration, motility and morphology. Sperm morphology is considered to be the most accurate predictor of prognosis (Coetzee *et al*, 1998; Ombelet *et al*, 1995; Kruger *et al*, 1988; Menkveld *et al*, 2001).

In order to treat male factor infertility, more advanced fertilization techniques including; intracytoplasmic sperm injection (ICSI), physiological intracytoplasmic sperm injection (PICSI®) and intracytoplasmic morphologically selected sperm injection (IMSI) have been developed. These will be discussed in section 1.4 Advanced Methods of Fertilization.

1.2 Infertility: A Global Overview

The clinical definition of infertility, as provided by the World Health Organisation, is “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” (World Health Organization, 2010).

1.2.1 Prevalence and Distribution

In 2012, a systematic analysis of 277 health surveys illustrated national, regional, and global trends in infertility prevalence since 1990 (Mascarenhas *et al*, 2012). In analysing demographic and reproductive health surveys, researchers used a consistent algorithm to calculate infertility. A 5-year exposure period based on union status, contraceptive use and the desire for a child was used a demographic measure of infertility. Live birth was used as outcome and biases were corrected for in instances of incomplete information. Little changes in infertility rates were found over two decades, apart from specific regions in Sub-Saharan African and South Asia, taking into account global population growth and a declining tendency in the preferred number of children per household. The aetiological causes of the reported trends were undetermined and further studies are needed to clarify causes (Mascarenhas *et al,* 2012). The consensus among publications is that the question of calculating a single global estimate of infertility prevalence is complicated by lacking data in developing countries as well as variability in demographic definitions of infertility, outcome measures used and inconsistent research settings (Dyer, 2009). According to Ombelet *et* al (2008) more than 70 million couples are believed to suffer from infertility worldwide, with the prevalence of primary and secondary infertility varying widely. Infection is cited as the most common cause of infertility among men & women in Africa with regional prevalence rates as high as 30-40% (Dyer *et al*, 2002).

The most common causes of female subfertility include; advanced maternal age, ovulatory abnormalities or complete anovulation, tubal obstruction or disease, uterine factor, and adhesions or endometriosis (Healy *et al*, 1994). Male factor subfertility may be the result of varicocele, obstructive azoospermia, excessive scrotal heat or testicular injury, presence of anti-sperm antibodies, a genetic factor or exposure to environmental factors such as pesticides or industrial chemicals (Whorton *et al*, 1977; der Kretser, 1997). Idiopathic subfertility, where there is no demonstrable physiological cause to explain failure to conceive, represents approximately 5-10% of the infertile population seeking treatment (Hewitt *et al*, 1985). A small proportion of couples are affected by a combination of both male and female factors (Adamson & Baker, 2003).

A vast body of literature exists to demonstrate an age-dependent decline in female fertility. A retrospective population-based study on women age 22-44 years in Denmark, Germany, Italy & Spain evaluated the associated risk of maternal & paternal age combined into a single variable in a multivariate logistic regression analysis. The reference group used was couples where both partners fall into the 20-29 years grouping. Results were adjusted for a number of factors including; reproductive history and country and reported a higher risk of miscarriage in women 35 years and older consistent with previous publications (de La Rochebrochard & Thonneau, 2002). Novel results included the observation that couples composed of a woman 35 years or older combined with a male partner of 40 years and over were at a significantly increased risk of all potentially adverse outcomes (de La Rochebrochard & Thonneau, 2002). It was concluded that the risk of adverse pregnancy outcome, particularly miscarriage, is greatest when both partners are of advanced age (de La Rochebrochard & Thonneau, 2002).

Fewer studies evaluating the possible adverse effect of advancing paternal age on reproductive capability are available (Broekmans *et al*, 2007). Advanced paternal age has also been associated with impaired neurocognitive outcomes during infancy and childhood based on finding drawn from the US Collaborative Perinatal Project (Saha *et al*, 2009). A number of outcome measures including various well-known intelligence and achievement scales designed for children were applied at 8 months, 4 years and 7 years. Analyses were examined after being adjusted for potential confounding factors and advanced paternal age was consistently associated with poorer test scores in all neurocognitive assessments except the Bayley Motor score at all age intervals (Saha *et al*, 2009). Conversely, a 2011 systematic review of existing literature included 10 studies and reported insufficient evidence to support an unfavourable effect of paternal age on ART outcomes (Dain *et al*, 2011). Further concerns regarding age associated male infertility have recently arisen following studies illustrating a high prevalence of isolated sperm DNA damage in older men (Das *et al*, 2013).

This list of causes for subfertility is by no means exhaustive and is only one of the many factors influencing the potential outcome of treatment. The success of any given infertility treatment is multifactorial, the diagnostic findings regarding the initial cause of subfertility, ovarian response to stimulation, number of oocytes harvested, quality of resulting embryos and endometrial receptiveness are some of the factors determining the likelihood of an ongoing pregnancy and live birth (Boivin *et al*, 2007).

1.2.2 Current Success Rates

The European Society of Human Reproduction and Embryology has published 14 monitoring reports on the results of medically assisted reproduction treatments including both assisted reproductive technology (ART) and intrauterine insemination (IUI) cycles initiated in Europe within various time periods. Trends and fluctuations are also discussed in each report. The most recent of such publications was published in *Human Reproduction* in 2014 and reported data from national registries of individual countries on European cycles initiated in 2010 (Kupka *et al*, 2014).

The participants included 31 European countries, 991 clinics and 550 296 ART treatment cycles. It should be mentioned that the findings are limited by a lack of standardized reporting method among European countries and authors have suggested results be interpreted with caution. Despite noting some fluctuations in the number of countries, reporting the overall number of initiated ART cycles has continued to increase with each new year. Pregnancy rates were comparable to that reported from 2009 (Kupka *et al*, 2014). In respect of different treatments, the following pregnancy rates were reported; IVF clinical pregnancy rates per aspiration and per transfer increased to 29.2% and 33.2% respectively. The corresponding clinical pregnancy rates following ICSI treatment also indicated an increase from 28.8% in 2009 to 32.0% in 2010. The pregnancy rate of frozen embryo cycles reported was 20.3% per thawing. The delivery rate of singleton, twin and triplet pregnancies, combined between IVF and ICSI cycles, were 79.4%, 19.6% and 1.0%, respectively. The total multiple delivery rate was therefore 20.6% compared with the 20.2% reported in 2009. Although only marginally lower than the multiples rate of 2009, the decline in multiples rate is likely due to a movement towards single embryo rather than multiple embryo (three or more) transfers. Over half a million ART cycles were reported in 2010 consistent with reports of consistently expanding numbers of ART treatments per year (Kupka *et al*, 2014).

A retrospective, cross-sectional survey on the utilization, effectiveness and safety of ART procedures performed globally from 2008 to 2010 was recently published in Human Reproduction (Dyer *et al*, 2016). Data from nearly 2500 ART clinics from approximately 61 countries was included. The International Committee for Monitoring Assisted Reproductive Technologies (ICMART) developed forms and methods for the processing of data aggregated by country. It was concluded that the global utilization and effectiveness of ART remained relatively constant with only a 6.4% increase in the number of aspirations between 2008 and 2010. There was an increase in the rate of single embryo transfers from 25.7% in 2008 to 30.0% in 2010 as well as a 27.6% increase in the number of FET cycles globally over the

period stated (Dyer *et al*, 2016). While the report accounts for approximately two-thirds of global ART activity, it is limited by the quality and completeness of submissions from individual countries. The South African Register of Assisted Reproductive Techniques (SARA) has also recently been implemented for the monitoring of ART activity in sub-Saharan Africa. The 2013 SARA report conveyed that fertilization rate per aspiration is comparable between IVF (95.3%) and ICSI (94.9%) (Dyer, 2013). Clinical pregnancy rates for IVF/ICSI have remained constant over the past 5 years but an increase in the multiple pregnancy rate from 14.4% in 2012 to 26.6% in 2013 has been reported. The increase in multiple pregnancy rate may be the result of better reporting of outcomes rather than a true increase but such a high multiples rate nevertheless motivates further efforts to move towards single embryo transfers in the future (Dyer, 2013).

1.2.3 Research to Improve Success Rates

In the field of assisted reproduction, research is often dedicated to what are considered the most relevant parameters in determining the outcome of infertility treatments. A large number of recent publications have focussed on the improvement of ongoing pregnancy and live birth rates while others aim to develop new techniques to reduce the frequency of high order multiple pregnancies, commonly associated with assisted reproduction treatments (Ombelet *et al*, 2005). In the quest to improve ART success ongoing research efforts include; the development of more sophisticated embryo and sperm selection techniques as well as strategies to enhance implantation rates, such as HA product usage.

a. Embryo selection

Widespread research exists to demonstrate the crucial nature of embryo selection in the treatment of patients wishing to conceive by assisted reproductive technologies. Embryologists employ various embryo selection strategies at different developmental stages, from oocyte to blastocyst stage, with the aim of identifying the most implantation competent embryo(s), with the highest potential to result in an ongoing singleton pregnancy, for transfer (Montag *et al*, 2013).

Embryo selection can be divided into two primary groups; invasive and non-invasive techniques (Kotze *et al*, 2013). Non-invasive selection techniques include morphological assessment of oocytes and embryos directly following aspiration and throughout the cleavage-stages of development leading to the formation of the blastocyst (Fisch *et al*, 2001). Blastocyst evaluation

includes grading of the expansion and appearance of both the trophectoderm cells and the inner cell mass (Alfarawati *et al*, 2011). Although all three parameters are important, the predictive strength of the trophectoderm grading is greater than that of the inner cell mass grading (Ahlstrom *et al*, 2011; Gardner & Lane, 1997). Pronuclear morphology may also be assessed although this technique is used mainly in research rather than informing the decision of which embryo to select for transfer in most private clinics (Balaban *et al*, 2001). Invasive procedures involve the biopsy of polar bodies, blastomeres or trophectoderm cells to enable the screening of the oocyte or embryo's genetic constitution for abnormalities (Tarin & Handyside, 1993). The timing of the first post fertilization mitotic division in early zygotes may also predict viability. Early cleavage has been studied extensively with numerous studies reporting that early cleavage is not only associated with increased developmental potential (Fenwick *et al*, 2002; Windt *et al,* 2004) and improved embryo quality (Fu *et al*, 2009) but with a greater likelihood of blastulation (Van Montfoort *et al*, 2004). A study including the analysis of 178 single embryo transfers reported a significantly higher clinical pregnancy rate (50%) after transfer of "early cleavage" embryos when compared with "no early cleavage" embryos (26.4%) (Salumets *et al,* 2003). Embryos were checked for early cleavage at 25-27 hours post insemination or ICSI and selection for transfer was based on day 2 embryo morphology and growth rate, not early cleavage (Salumets *et al, 2003).* Current embryological dogma has been called into question by researchers evaluating the relationship between ploidy status and blastocyst formation of fast cleaving embryos (Luna *et al*, 2008). A retrospective study evaluating the blastulation, implantation, pregnancy and birth rates resulting from fast embryo transfers reported a significantly greater blastulation rate in the intermediate (72.7%) and fast cleaving (54.2%) groups when compared with slow cleaving (38%) groups (Luna *et al,* 2008). Similarly, a reptrospective study has also reported a negative association with abnormal early cleavage (Phan *et al*, 2014). A study that evaluated 1557 embryos from 203 ICSI cycles with array comparative genomic hybridization (a-CGH), the relationship between embryo morphology and development and chromosomal status was evaluated. Day 3 embryos were biopsied and all 23 chromosome pairs were analysed. The researchers reported greater incidence of chromosomal abnormalities in both slow- and fast cleaving embryos when compared with embryos that followed traditional cleaving patterns consistent with days post insemination (Phan *et al*, 2014). The assessment of embryos through the metabolomic profiling of embryo culture media has also been investigated as a useful adjunct to current morphological embryo assessment (Botros *et al*, 2008).

Invasive procedures involving the biopsy of polar bodies, blastomeres or trophectoderm cells enable the screening of the oocyte or embryo's genetic constitution for chromosomal

abnormalities such as aneuploidy as well as many monogenic diseases (Mastenbroek *et al*, 2007). Pre-implantation genetic screening (PGS) involves the use of comparative genomic hybridization (CGH) or whole genome amplification (WGA) to identify the presence of genetic abnormalities of single disaggregated embryonic cells (Voullaire *et al*, 1999). PGS was reported by Voullaire *et al* in 1999 following their modification of a technique used to screen the whole genome's DNA in tumours developed by Kallioniemi *et al* (1994). CGH has since been adapted to be more efficient and thorough in its screening and is presently referred to as array-CGH (Phan *et al*, 2014). Embryos with chromosomal abnormalities are causally linked with poor implantation rates and the selection of embryos based on normal chromosomal constitution therefore results in the improvement of both implantation and ongoing pregnancy rates in ART facilities (Gianaroli *et al*, 1997; Phan *et al*, 2014).

The analysis of transcriptomes and proteomes obtained from biopsied embryonic tissue has also proven useful in further understanding the coordination of the series of complex developmental events leading to blastocyst stage but its usefulness in a routine clinical setting has not yet been established (Montag *et al*, 2013).

A number of studies including a recent study using time-lapse monitoring by Kirkegaard *et al.* (2012) have confirmed the importance of maintaining the integrity of the embryo throughout development and have detailed the possible negative effects of blastomere removal on continuing embryo development (Cimadomo *et al*, 2016). In lieu of this, recent focus has shifted to the improvement and establishment of new non-invasive techniques where the investigation itself has no possible effect on the embryo (Kotze *et al,* 2012).

A novel selection tool called time-lapse imaging involves the incubation of embryos in a specialized incubator with built in camera that enables the recording and retrospective analysis of key developmental activity known as morphokinetics (Desai *et al*, 2014). This technique has been used to classify the risk of aneuploidy in respective embryos allowing a ranking of embryo competency and further assisting the process of embryo selection (Campbell *et al*, 2013; Costa-Borges *et al*, 2016). Time lapse has recently also enabled the study of various cleavage anomalies and multinucleation (Desai *et al*, 2014; Liu, 2016). A 2016 prospective, randomized, controlled trial assessed whether the addition of time-lapse morphokinetics in the selection of embryos for transfer improved pregnancy rates. The study included a total of 235 patients, 116 of which were randomized to conventional once-daily morphologic embryo screening (CS) and 119 to additional time-lapse kinetic monitoring (TLM). The intrauterine clinical pregnancy (TLM

68% vs. CS 63%) and implantation rates (TLM 51% vs. CS 45%) were not statistically significantly different (Goodman *et al*, 2016). The Biennial review of infertility explains that although over 20 morphokinetic markers have been identified using TLM, these biomarkers have not been properly validated in prospective randomized controlled studies and their application remains experimental (Bormann *et al,* 2015). An algorithm, the KIDScore, has also been developed to enable the ranking of cleavage stage embryos according to their blastocyst formation potential. The algorithm was based on the retrospective evaluation of 3275 embryos transferred on Day 3, conducted in 24 separate clinics, and is reported to have superior predictive power in comparison with other available blastocyst prediction algorithms (Petersen et al, 2016).

b. Sperm selection

Sperm selection techniques for use during ICSI have also evolved to enable the visualization of sperm under higher-magnification so that morphology can be assessed using the IMSI technique (Boitrelle, 2014). The physiology of sperm can also be assessed with the use of new hyaluronic acid (HA) based products such as PICSI® dishes (Huszar *et al*, 2006).

Other sperm selection methods less commonly used are annexin VI bead [MACS] columns and zona pellucida (ZP) selected sperm (Liu *et al*, 1989, Liu *et al* 2011). Spermatozoa preparation by magnetic activated cell sorting (MACS) eliminated apoptotic spermatozoa based on the presence of externalized phosphatidylserine residues (Dirican *et al*, 2008). The sorting of spermatozoa with MACS prior to gradient centrifugation prepatation resulted in greater percentages of morphologically normal spermatozoa as well as improving cleavage and pregnancy rates in oligoasthenozoospermic ART cases (Dirican *et al*, 2008). A prospective, randomized, triple-blinded trial in Spain evaluated the extent to which removing presumptive apoptotic sperm cells from frozen samples affects sperm quality. Other measured outcomes included the fertilization, cleavage, implantation and pregnancy rates following ICSI with MACS sorted spermatozoa. A total of 237 couples undergoing ICSI cycles with donor ova were included and no statistically significant improvements were observed in any of the measured outcomes (Romany *et al,* 2014).

In a study with 106 couples undergoing ICSI treatment with ejaculated sperm the use of ZP-bound spermatozoa for ICSI produced higher embryo quality rates and significantly improved implantation rates in comparison with conventional ICSI (Liu *et al*, 2011). A 2016 study

confirmed that ZP-bound spermatozoa used for ICSI significantly improved embryo quality rates although fertilization and cleavage rates remained unchanged (Jin *et al*, 2016).

c. Improved Implantation Potential

One of the many remaining research questions among professionals in the ART community is the matter of supporting successful implantation following embryo transfer, regardless of day of transfer. The concept of endometrial receptivity and the need for synchronization between the embryo and the endometrium remains widely debated among professionals in the reproductive medicine community. The process of implantation is multivariate and requires a reciprocal dialogue between the endometrium and the transferred embryo(s) (Bergh & Navot, 1992). The timing of embryo transfer in relation to embryonic development and maturity of the endometrial lining is therefore crucial in the determination of outcome. Studies on the peri-implantation period have enabled the detection of embryonic signalling between cycle days 20 and 24, the presumed window of implantation (Bergh & Navot, 1992). In a study by Bergh & Navot (1992) 33 pregnancies resulted in deliveries following 75 embryo transfers. Embryos of the same age were transferred on different days within the day 15 to day 19 window. These days each represent different stages of endometrial maturation with unique hormonal and histological profiles. Embryonic age and endometrial maturation were compared with individual hCG linear regression models and day 19 was found to have the steepest slope of hCG detection relative to days 15 and day 16 (Aplin, 2000). It was concluded that implantation depends on embryonic age irrespective of endometrial maturation between cycle days 20 to 24, the presumed window of implantation (Bergh & Navot, 1992). The adequacy of induced endometrial cycles following hormonal manipulation has been experimentally evaluated in terms of various histological and hormonal criteria. A particular set of histological, hormonal and morphological criteria are used to classify endometrial normalcy and receptivity to embryos (Aplin, 2000; Sharkey & Macklon, 2013).

The Cochrane Library published a review of Assisted Reproductive Technology in Cochrane Reviews in 2015. The main results of the review organized interventions categorically as either effective, promising, ineffective, possibly ineffective and those where no conclusions were possible due to lack of evidence (Farquhar *et al*, 2015). Interventions classified as effective can be observed in Table 1.

Table 1: Effective interventions in ART treatment.

Pre-ART and adjuvant strategies	• Endometrial injury • Growth hormone use • Metformin use in PCOS patients • Surgical treatment for tubal disease
Down-regulation with agonist or antagonist	• GnRHa protocols for pituitary suppression • GnRH antagonist treatment • Long term pituitary down-regulation before IVF
Ovarian stimulation	• Recombinant versus urinary gonadotropin use • Long-acting FSH versus daily FSH
Ovulation triggering	• Recombinant versus urinary hCG for final oocyte maturation • GnRHa versus hCG for triggering in antagonist cycles
Oocyte retrieval	• Individualized pain relief during oocyte retrieval
Laboratory phase	• Low oxygen for embryo culture
Embryo transfer	• Ultrasound versus “clinical touch” for catheter guidance • Adherance compounds (hyaluronic acid) in transfer media • Single embryo transfer versus double embryo transfer
Luteal phase support	• Progesterone as best method of luteal support
Prevention of ovarian hyperstimualtion syndrome	• Intravenous hydroxyethyl • Cabergoline • GnRH antagonist protocols • GnRHa versus hCG for oocyte triggering in antagonist cycles

1.2.4 Hyaluronic Acid/ Hyaluron products

Hyaluronic acid (Hyaluronan, HA) is a naturally existing macromolecule produced by the human cumulus cells present in varying concentrations throughout the female reproductive tract and the endometrium (Salamonsen *et al,* 2001). HA is a unique linear glycosaminoglycan (GAG) with a negative charge that attracts water and allows significant expansion of its solvent domain beyond the actual polymer volume (Salamonsen *et al,* 2001). This property plays a role in enabling cell-cell contact in a number of biological processes while HA also provides a matrix for cell migration. Additionally, HA facilitates cellular signalling during implantation by binding to receptor CD44 expressed on both the embryo and in the stroma of the endometrium (Salamonsen *et al,* 2001). The distribution of HA varies as the endometrium undergoes extensive remodelling throughout the various phases of the menstrual cycle. These changes in HA deposition have been studied using a highly specific binding peptide for histochemical localization. The HA deposition in the stromal compartment was found to peak during the mid-proliferative (days 5-10) and mid-secretory (days 19-23) phases of the cycle. In contrast, the deposition of HA around blood vessels was found to remain constant throughout the cycle (Salamonsen *et al,* 2001). HA has been shown to increase both cell-to-cell and cell-to-matric adhesion known to promote successful implantation of the embryo (Huszar *et al*, 2007). The viscosity of a HA solution is believed to prevent expulsion during the transfer process while facilitating the integration of embryos into the viscous intrauterine secretions. Studies on mice comparing the use of embryo transfer medium with and without HA resulted in improvement in both implantation and foetal development rates where hyaluronic acid was present (Turley & Moore,1984).

Several hyaluronic acid (HA) based products aiming to improve overall ART outcomes are currently available on the market including PICSI® dishes and EmbryoGlue® (discussed in section 1.4 Advanced Methods of Fertilization and section 1.2.3 Research to Improve Success Rates).

SpermSlow is a product that assists the sperm selection process by slowing the movement of spermatozoa to allow better visualization of morphology where good morphology is associated with functional maturity, fertilization capability and normal chromosomal constitution (Sun *et al*, 2006). The use of SpermSlow has been suggested as a more natural, and biodegradable, substitute for PVP conventionally used for ICSI cases (Huszar, 2012). A prospective randomized control trial comparatively evaluated the use of PICSI® dishes and Sperm Slow in sperm hyaluronic binding selection for fertilization by intra-cytoplasmic sperm injection. It was found that there is no significant difference in clinical efficiency of the two systems in selecting

hyaluronic acid bound spermatozoa (Parmegiani *et al*, 2012). A retrospective comparison between PVP-ICSI and HA-ICSI illustrated a significant improvement in both embryo quality and implantation rates associated with spermatozoa physiologically selected with HA-ICSI (Parmegiani *et al*, 2010a).

EmbryoGlue® (EG) by Vitrolife is an embryo transfer-specific medium containing a unique combination of hyaluronic acid and recombinant albumin. The hyaluron contained in EG acts as a specialised adherence compound supporting the implantation of a transferred embryo in the endometrium (Simon *et al*, 2003). Initial studies on mice comparing the use of embryo transfer medium with and without HA resulted in an improvement in both implantation and fetal development rates where hyaluronic acid was present (Turley & Moore, 1984). (Discussed in section 1.2.3 Research to Improve Success Rates).

Details of the proposed working mechanism of EG have been discussed in subsection 1.2.3, “Research to Improve Success Rates” but briefly it has known been to increase both cell-to-cell and cell-to-matric adhesion necessary for effective communication between the embryo and endometrium during implantation (Huszar *et al*, 2007). Additionally, the viscosity of a HA solution is believed to prevent expulsion during the transfer process while facilitating the integration of embryos into the viscous intrauterine secretions.

In 2005, a study designed to evaluate the usefulness of EG in improving pregnancy rates included 310 fresh embryo transfers, 154 of which were transferred following incubation in the HA-enriched medium. Possible confounding factors such as age, duration of infertility, previous IVF cycles, total gonadotropin dose, oocyte number and number of embryos transferred were similar in the control and treatment groups. The resulting pregnancy rates were not significantly different and they concluded the use of EG in fresh transfers did not improve resulting pregnancy rate (Ravhon *et al*, 2005). A prospective randomized study that followed showed an overall increase in implantation and clinical pregnancy rates where EG was favoured above conventional transfer medium (Urman *et al.,* 2008). Interestingly, the use of EG had the greatest beneficial effect on patients who had previously failed cycles and in women with poor-quality embryos (Urman *et al.,* 2008). A meta-analysis published by the Cochrane Collaboration research network in 2010 concluded that high concentrations of HA in a transfer medium (EmbryoGlue® by Vitrolife) resulted in evidence of treatment effects for clinical pregnancy rate and multiple pregnancy rate, with higher rates in the hyaluronic acid groups (Bontekoe *et al*, 2010). HA groups are labelled either as high (0.5 mg/ml), low (0.125mg/ml) or no HA (0.0 mg/ml) (Bontekoe *et al*, 2010).

The increase in multiple pregnancy rates requires further studies with single embryo transfer as the policy of transferring more than one embryo is a possible confounder. No evidence of an effect on live birth rate was found (Bontekoe *et al*, 2010). The review article that followed the Cochrane review mentioned that low concentrations of HA in transfer medium has no effect on clinical pregnancy rate and that live birth rate (LBR) was found not to have improved significantly (Bontekoe *et al*, 2010). LBR had not been the end point for many of the studies included and this was thought to explain the lack of evidence supporting higher LBR (Bontekoe *et al*, 2010). A 2014 meta-analysis on almost 10 000 embryo transfers carried out at various Japanese clinics further confirmed the findings of the Cochrane report evaluating the use of high concentrations of HA in embryo transfer medium (Hashimoto *et al*, 2014). The meta-analysis concluded that the use of EmbryoGlue® as transfer medium resulted in a significant increase in both implantation and pregnancy rates. Miscarriage rates were not significantly different (Hashimoto *et al*, 2014).

In 2014, the Cochrane Collaboration published an update of their previous meta-analysis also entitled “Adherence compounds in embryo transfer media for assisted reproductive technologies (Review)”. The updated review included 16 truly randomized controlled trials with a total of 3898 total participants. Out of those included, 14 studies reported CPR as endpoint and 6 studies also included LBR. The use of EG for embryo transfer, when compared with the use of culture medium containing little or no hyaluronic acid, resulted not only in an improvement of successful implantation rates but also in an overall increase of 8% live birth rates, regardless of embryo transfer day (Bontekoe *et al.,* 2014).

However, a 2015 cohort study performed on 229 retrospectively enrolled patients found no significant improvement in ART outcomes after routine use of EG. Decreased abortion (15.8% vs. 19%) and increased multiple pregnancy rates (20.6% vs. 15.6%) in the study group were not statistically significant (Safari *et al,* 2015). Patients in both groups were comparable in terms of etiology of infertility, history of implantation failure and rates of good quality embryos to limit confounding variables (Safari *et al,* 2015). Additional abstracts from oral and poster presentations at the American Society for Reproductive Medicine (ASRM) scientific congress & expo were published by Fertility and Sterility in September 2013 (Hambiliki *et al*, 2010; Said *et al,* 2013; Singh *et al*, 2015). A study from the Center for Reproductive Medicine of New Mexico, Albuquerque including 179 frozen embryo replacement cycles reported that the use of EG as post-thaw culture and transfer medium did not improve the measured clinical outcomes in frozen embryo replacement cycles (Said *et al,* 2013). The measured outcomes included clinical and ongoing pregnancy, and implantation rate per embryo transferred (Said *et al,* 2013). Another study investigating the addition of HA to embryo transfer media in cleavage-stage

frozen embryo transfers resulted in increased biochemical pregnancy and implantation rates but live birth rate remained unchanged (Hambiliki *et al*, 2010).

A prospective case-control study conducted at the assisted reproductive centre of a tertiary care hospital involved 84 women undergoing fresh non-donor IVF or ICSI cycles (Singh *et al*, 2015). In the control group (*n*=42), embryos were transferred to conventional blastocyst culture medium and those in the treatment group (*n*=42) were transferred into 50 µL of EG for 10 min prior to transfer inside the uterine cavity. In an effort to limit possible confounding factors the exclusion criteria included women age >35 years, those with poor ovarian reserve, and possible causes of failure of implantation such as diabetes mellitus, hypertension and autoimmune diseases. The resulting clinical pregnancy rate in the treatment group was 7% higher than that of the control group; the difference was not statistically significant (Singh *et al*, 2015). A significant difference (p=0.04) in clinical pregnancy rate was observed in a subset (n=12) of treatment patients who had previous failed ART cycles. In the treatment group, 50% [6/12] of patients achieved successful implantation where none of the patients [0/11] in the control group did (Singh *et al*, 2015).

The use of EG remains controversial among ART professionals, as it is difficult to conclude the favourable role of EG in treatment cycles of patients with good prognosis. The improvement in implantation rates observed in patients with previous failed cycles and recurrent implantation failure demands further investigation.

The first phase of this study is a retrospective comparison between conventional ICSI and PICSI®; on various treatment outcomes. The second phase is a prospective study to determine the usefulness of a different HA product, EG, in a unique clinical setting.

1.3 The Process of Fertilization

1.3.1 Capacitation

An oocyte must undergo meiotic maturation and be in metaphase II in order to fertilize successfully. Similarly, a spermatozoon must also undergo a series of biochemical and physiological changes before it is able to initiate the process of fertilization (Küpker *et al*, 1998). *In vivo,* capacitation of the spermatozoon occurs upon exposure to the high quantities of extracellular ions. The female reproductive tract contains co-operative factors, such as Ca^{2+} and Na^{+} ions that drive the capacitation process resulting in functionally mature, mobile and fertile sperm (Suarez & Pacey, 2006). During capacitation, the functional coupling of signal transducing pathways destabilizes the spermatozoon's plasma membrane. The removal and mobilization of several surface compounds, such as cholesterol and glycoproteins, also drives the capacitation process (Bailey, 2010). These biophysical membrane changes result in a hyperpolarization of membrane potential followed by the influx of extracellular bicarbonate. The increased intracellular bicarbonate stimulates adenyl cyclase resulting in increased production of signalling molecule Camp (Bailey, 2010). Cells respond by the rapid influx of extracellular calcium. During the capacitation process, membrane fluidity increases, the net surface charge is altered and there is the potential for fusion of the acrosomal membrane. In its capacitated state, the spermatozoon is able to respond to molecular signals from the oocyte and the cumulus-oocyte complex (COC) that surrounds it (Guraya, 2000). The capacitation process can also be induced with specialized synthetic media in an *in vitro* setting (Shih *et al*, 2016).

1.3.2 Hyperactivation

The penetration of the COC causes changes in the frequency and pattern of the spermatozoon's flagellar movement. The frequency of flagellar movement increases and patterns of movement become three-dimensional. This hyperactivation of motility aids the spermatozoon in penetrating the cumulus mass as it propels itself towards the oocyte (Ickowicz *et al*, 2012).

1.3.3 The Acrosome Reaction

The acrosome reaction is an exocytotic process initiated by intracellular signals following spermatozoon interaction with the three glycoproteins, ZP1, ZP2 and ZP3, of the oocyte zona pellucida (ZP) (Brucker & Lipford, 1995). The influx of calcium ions and cytoplasmic alkalinisation during adhesion to the ZP are key intracellular signals responsible for the initiation

of the acrosome reaction and activation of receptors in the sperm head. During the acrosome reaction, the plasma membrane fuses with the outer acrosome membrane releasing the enzymatic contents of the acrosome that further aid in oocyte penetration and fusion (Brucker & Lipford, 1995). Hyaluronidase is one of the enzymes present during this reaction, it aids in the digestion of the cumulus complex that surrounds the oocyte (Ickowicz *et al*, 2012). Upon reaching the PVS the fusion receptors of the spermatozoon will begin to interact with the oolemma of the oocyte (Allen & Green, 1997).

1.3.4 Sperm-Oocyte Binding and Fusion

Located between the inner acrosomal membrane and the plasma membrane in the posterior region of the sperm head lies the equatorial segment of the spermatozoon, a key participant in the fusion reaction (Kaji & Kudo, 2004). Once this equatorial segment binds with microvilli covering the surface of the oocyte, except in the region of its meiotic spindle, the sperm nucleus is released into the ooplasm. Oocyte-activating proteins, such as oscillin that trigger calcium oscillations, are released during fusion when fertilization occurs normally (Wakai & Fissore, 2013). During ICSI the fusion of the membrane is bypassed and damage to the sperm plasma membrane, prior to injection, instead allows sperm-associated oocyte activating factor (SAOAF) to exit the spermatozoon (Dozortsev *et al*, 1997). The disruption of the plasma membrane by immobilizing the spermatozoon before ICSI is necessary for fertilization to occur successfully (Yanagida *et al*, 2001).

1.3.5 Chromatin structure, Fragmentation & Abnormality

Spermatogenesis is the development of mature spermatozoa from primordial germ cells (Gunes *et al*, 2015). The functional maturity and fertilization capability of a spermatozoon are determined by the ability of its DNA repair mechanisms to maintain genomic integrity as it matures (Gunes *et al*, 2015). The three main origins of DNA damage in the male germline include; endogenous or exogenous reactive oxygen species (ROS) involving abasic sites, base modifications, single-strand and double-strand breaks and DNA proteins cross links (Torregrosa *et al*, 2006). Defects in sperm chromatin packaging can interfere with the protamination of sperm chromatin, the crucial replacement of histones by protamines, which protects its genome from oxidative damage in the harsh environment of the female reproductive tract (Ward, 2010). Protoamination also assists in the compaction required to fit the male haploid genome into the nucleus of each spermatozoon (Torregrosa *et al*, 2006). The final nuclear shape acquisition of the spermatozoon assists in the safe

and accurate transmission of the genetic information from the male to the oocyte in a usable format prior to fertilization (Agarwal & Said, 2003).

A study examining sperm chromatin defects by flow cytometric sperm chromatin structure assay concluded that the probability of achieving pregnancy declines as a direct function of the percentage of sperm with defects in their chromatin structure (Spano *et al,* 2000). Any DNA damage or abnormality may result in male infertility. Sperm with abnormal morphology may have functional receptor defects that would hinder their ability to bind with the glycoproteins on the surface of the oocyte (Agarwal & Said, 2003).

1.4 Advanced Methods of Fertilization

1.4.1 The Development of intracytoplasmic sperm injection (ICSI)

ICSI technique, is a micromanipulation fertilization technique routinely employed in modern assisted reproduction facilities globally. The technique was first applied to human gametes in the early 1990s (Tarlatzis & Bili, 1998) but its use was described in sea urchin studies as early as 1962 (Hiramoto, 1962). Live spermatozoa of three unique sea urchin species were microinjected into oocytes from the same respective species but no activation was reported in microinjected unfertilized eggs. Injected eggs were however successfully fertilized by insemination from their exterior (Hiramoto, 1962). The first mammalian application of ICSI, resulted in the live birth of 6 undersized experimental mouse fetuses (Lin, 1966). Ten years thereafter, human and hamster spermatozoa were microinjected into hamster oocytes but few oocytes survived the procedure (Uehara & Yanagimachi, 1976).

Several alternative fertilization techniques were developed as it became apparent globally that the percentage of normally fertilized oocytes and number of embryos generated by conventional IVF was significantly lower when semen characteristics were below reference ranges in terms of concentration, motility and morphology (Tournaye *et al*, 2002).The first experimental technique was partial zona dissection (PZD). Sperm could easily enter the oocyte through the artificial opening to reach the oolemma but results were found to be unpredictable and therefore unreliable (Palermo *et al*, 1992). Subzonal insemination (SUZI) technique, involving the microinjection of few motile sperm through the zona pellucida (ZP) into the perivitelline space (PVS), soon followed. With approximately 20% normal fertilization of oocytes following SUZI, the majority of patients had only one or two embryos available for transfer per cycle. It was concluded that the pregnancy and live birth rates following both PZD and SUZI techniques were too low to implement in routine clinical practice (Palermo *et al*, 1992). In 1992, the Lancet published the first pregnancy and live birth data of treatment cycles where embryos were generated and replaced following the ICSI technique in humans (Palermo *et al*, 1992). Previous success with ICSI treatment had been exclusively in animal studies. IVF researchers in the USA and Singapore also conducted ICSI trials and although they observed greater fertilization rates than was demonstrated in PZD and SUZI and transferred cleavage stage embryos, no pregnancy was established (Lanzendorf *et al*, 1988).

ICSI is effective in the treatment of a variety of male factors but in order to generate a normally fertilized embryo the spermatozoon selected for microinjection into an oocyte must have both a functional genome and a centriole (Bhattacharya *et al,* 2001). ICSI is usually performed with

ejaculated sperm when indicated for by poor motility, low concentration or poor morphology as well as oligoasthenoteratozoospermia (OAT), failed fertilization in IVF or even the presence of antisperm antibodies (Bhattacharya *et al,* 2001). In cases of obstructive azoospermia, such as the congenital absence of the vas deferens (CBAVD), or disturbances in spermatogenesis, sperm may be retrieved from the epididymis and testis. Testicular or epididymal biopsy may also be carried out for patients with non-obstructive azoospermia, post vasectomy patients, patients with severe impotence or paraplegic patients (Monga *et al*, 1999). Depending on the physiology of the male partner, microsurgical epididymal sperm aspiration (MESA), percutaneous sperm aspiration (PESA) or testicular sperm extraction (TESE) open biopsy technique can be performed (Friedler *et al*, 1997). ICSI is also the preferred method of fertilization in cases involving human immunodeficiency virus and hepatitis C virus-serodischordant couples with infected male partner to reduce the risk of transmission (Mencaglia *et al*, 2005).

1.4.2 10 Years of ICSI: A Review

In 2002, 10 years after the first live birth resulting from ICSI, a Serono Symposia International Conference on ICSI was held at the Vrije Universiteit Brussel (VUB). Healthcare professionals from around the globe gathered to review outcomes of several thousand ICSI cycles and to compare them with conventional *in-vitro* fertilization cycles. In terms of ovarian oocyte retrieval, fertilization, cleavage and implantation rates, ICSI results were comparable to conventional IVF as summarised in Table 1.1 (Van Steirteghem, Devroey & Liebaers, 2002).

Table 1.1 Comparing IVF and ICSI Outcomes in Europe in 1998.

	IVF	ICSI
Cycles started	51 471	34 576
Aspirations	46 474	33 133
Aspirations/ cycle started (%)	90.3	95.8
Embryo transfers	40 980	30 460
Embryos transferred/ aspiration (%)	88.2	91.9
Pregnancies	11 384	8 419
Pregnancies/ embryos transferred (%)	27.8	27.6
Deliveries	8 950	6 510
Deliveries/ cycle started (%)	17.4	18.8

During the period reviewed, ICSI accounted for approximately 40% of treatment cycles according to reports published by IVF/ICSI registries. In 2002, a randomised controlled trial comparing IVF and ICSI for the treatment of non-male factor infertility followed the observation that there was a global tendency to use ICSI as the standard fertilization method even when sperm parameters were within normal reference ranges (Bhattacharya *et al,* 2001).

1.4.3 Physiologic Intracytoplasmic Sperm Injection (PICSI®)

Physiologic intracytoplasmic sperm injection (PICSI®) involves the use of a specialised culture dish during the fertilization procedure (Huszar *et al*, 2007). A PICSI® dish has three microdots of hyaluronan (HA) attached to the bottom surface of its interior (Appendix VII). These HA dots provide an additional parameter whereby normal spermatozoa can be selected for injection (Huszar *et al*, 2007). Dr. Gabor Huszar of the Yale School of Medicine patented the PICSI® sperm selection devices after two decades of focussed research on potential biochemical markers of sperm maturity and development. A biotech company, Biocoat, announced the FDA's clearance of the new product, PICSI® dishes, in 2006 (Huszar *et al*, 2007). PICSI® dishes function on the principle that the HA receptors of functionally mature sperm will bind to areas of the dish coated with HA thereby improving the process of sperm selection by adding a basis for the assessment of sperm maturity (Huszar *et al*, 2007; Prinosilova *et al*, 2009).

During spermiogenesis the hyaluronic acid binding sites on the surface of the spermatozoa are formed as the result of plasma membrane remodelling (Prinosilova *et al*, 2009). According to research, spermatozoa that are able to bind to solid-state hyaluronic acid have a chromatin structure with high DNA chain integrity associated with fertilization competence and normal chromosomal constitution (Yagni *et al*, 2010). Another 2010 study also concluded that the selection of spermatozoa using the PICSI® method leads to a higher number of spermatozoa without DNA fragmentation and resulted in a relative improvement of resultant embryo quality (Parmegiani *et al*, 2010a).

The design of the study included three prospective studies where the main measured outcomes included; assessments of the DNA fragmentation rate and nuclear morphology of HA bound spermatozoa in comparison with non-bound spermatozoa. The authors reported reduced DNA fragmentation and improved nucleus normalcy compared with non-bound spermatozoa. The injection of bound spermatozoa also significantly improved both the development and quality of embryos in a randomized study (Parmegiani *et al*, 2010b). However, a 2014 Cochrane review of advanced sperm selection techniques for assisted reproduction included the evaluation of two randomized controlled trials with a total of 581 patients. The concluding remarks published in a summary of their findings stated that current evidence was insufficient to permit the evaluation of advanced sperm selection strategy effectiveness in ART. No statistically significant difference was reported between groups in terms of the following measured outcomes; fertilization rate, pregnancy rate, cleavage rate, embryo quality, implantation rate, spontaneous abortion and live birth rate (McDowell *et al*, 2014).

A systematic review and meta-analysis searched *PubMed* and *Cochrane* for studies, published up to June 2015, describing the clinical outcome of ICSI cycles in which spermatozoa were selected based on their ability to bind to hyaluronic acid (Beck-Fruchter *et al*, 2016). The only restriction applied to the search was species- humans. Only full-text articles, with control group and specified sperm selection technique were included. The Downs and Black Checklist (Downs & Black, 1998) was used to assess the quality of the seven studies included and meta-analysis was done where data was available (Ciray *et al*, 2008; Van Den Bergh *et al*, 2009; Parmegiani *et al*, 2010a; Parmegiani *et al, 2010b;* Choe *et al*, 2012; Majumdar & Majumdar, 2013; Worrilow *et al*, 2013). The fertilization rate could be calculated and compared in all seven studies, with more than 9700 injected oocytes in total. Meta-analytic pooling of all data indicated no association between HA based sperm selection technique and fertilization rate. Subgroup meta-analysis of the prospective studies also indicated no improvement in fertilization rate where HA binding was used to select sperm. Clinical pregnancy rate was calculated per cycle started and included nearly 1300 ART cycles. Meta-analytic pooling of data again revealed no association between sperm selection technique and pregnancy rate. Secondary outcomes measured included cleavage rate, which showed a significant difference in favour of conventional ICSI. Embryo quality, provided in only two studies from the same research group, demonstrated an improvement in embryo quality in the HA binding group. Miscarriage rates were comparable in conventional ICSI and PICSI® groups. Live birth rate was reported for three studies and meta-analysis showed no difference between the two groups. The available clinical literature simply does not support the use of HA binding assays in all ICSI cycles and the identification of patients that may benefit from PICSI® technique requires further investigation (Beck-Fruchter *et al*, 2016).

1.4.4 Intracytoplasmic Morphologically Selected Sperm Injection (IMSI)

During conventional ICSI, spermatozoa are viewed and selected under 400 times magnification (Antinori *et al*, 2008). At this magnification, spermatozoa with subtle nuclear morphological malformations carrying various structural abnormalities may be classified as normal. The described limitations of ICSI were alleviated by a method of motile sperm organellar morphology examination (MSOME) at 1,500 times magnification-introduced by Bartoov *et al* in 2001. An optical magnification of up to 1,500 times allows real time fine nuclear morphology assessment on motile spermatozoa (Bartoov *et al*, 2001). The high power differential interference contrast optics of an inverted microscope may be further enhanced by digital imagining to provide up to 6,000 times magnification (Bartoov *et al*, 2001). Oocyte microinjection using MSOME criteria is referred to as Intracytoplasmic morphologically selected sperm injection (IMSI). A normal nucleus has been defined as having an oval shape with

smooth configuration and less than four percent occupied by vacuoles. A nucleus comprised of greater than four percent vacuoles likely indicates poor chromatin integrity. Specific malformations indicating structural subcellular sperm abnormalities may involve the acrosome, the postacrosomal lamina, the nucleus, the neck, tail and the mitochondria (Bartoov *et al*, 2002). Acrosomal abnormalities include a partial or vesiculated acrosome, or spermatozoa may entirely lack an acrosome and postacrosomal lamina. Nuclear abnormalities may be identified as being undersized, enlarged, short or locally disordered (Berkovitz *et al,* 2005). Immaturity may be indicated by the presence of a cytoplasmic droplet in the neck region. The absence of a tail, a coiled or broken tail, multiple tails or an abnormally short tail relative to head and midpiece size is also considered abnormal (Berkovitz *et al,* 2005).

Matched control comparison studies have found that the most important factor influencing ICSI outcome is the presence of a morphologically normal sperm nucleus (Bartoov *et al*, 2002; Berkovitz *et al,* 2005 & 2006). A prospective randomized trial further assessed the usefulness and possible advantages of the IMSI technique over conventional ICSI. Treatment group outcomes were compared in terms of pregnancy, miscarriage and implantation rates and found that IMSI resulted in a significantly ($p=0.004$) higher pregnancy rate (39.2%) over ICSI (26.5%) when applied to cases of severe male factor infertility (Antinori *et al*, 2008). Patients with two or more failed treatment cycles had a pregnancy rate of 29.8% following IMSI versus a 12.9% pregnancy rate following ICSI ($p=0.017$). Miscarriage rates were also significantly reduced in the same subgroup following IMSI treatment (17.4% versus 37.5%) (Antinori *et al*, 2008). The clinical outcomes of the IMSI treatment group suggest that IMSI may improve IVF success rates in a selected group of patients with male factor infertility. However, a randomized sibling-oocyte study including 350 ICSI cycles to alleviate male infertility was one of a number of studies that found no difference in oocyte fertilization rate nor in embryo development between IMSI and conventional ICSI cycles (De Vos *et al*, 2013). A literature review of MSOME and IMSI techniques included twelve years' worth of data (Setti *et al,* 2013) and concluded that when conventional ICSI was compared with IMSI outcomes, IMSI consistently outperformed ICSI in terms of the percentage of top-quality embryos generated, implantation and pregnancy rates as well as reducing miscarriage rates (Setti *et al*, 2010). A Cochrane Review evaluated only truly randomized controlled trials (RCT) comparing ICSI and IMSI and concluded that results from RCTs do not support the clinical use of IMSI (Teixeira *et al*, 2013). The evidence for improved clinical pregnancy was considered to be of very low quality and no evidence of effect on live birth or miscarriage rates was found (Teixeira *et al*, 2013).

1.5 Aim & Objectives

Aim

The aim of this study was to evaluate two HA products: PICSI® dishes (MidAtlantic Devices, Origio, Harrilabs, South Africa) and EmbryoGlue® (EG) (VitroLife, Kat Medical, South Africa) at the Drs Aevitas Fertility Clinic, Pinelands.

Objectives

A **retrospective** record review evaluated the ART outcomes of patients treated with PICSI® in comparison with traditionally employed ICSI and IVF techniques [2013-2015].

Primary objective
To compare the OPR between the PICSI and ICSI groups using the IVF patient group as control.

Secondary objective
To compare the fertilization rate, embryo quality rate [day 2, 3 and 5] and miscarriage rate between the PICSI and ICSI groups using the IVF patient group as control.

- patient demographics (female age, number of oocytes aspirated, embryo quality and number of embryois transferred) were included in the analysis.

This comparison will allow it to be determined whether there is a statistically significant improvement in ART outcome in Drs. Aevitas Fertility Clinic, when PICSI® dishes were used to select spermatozoa for injection.

A **prospective** study was done to evaluate the ART outcomes of patients who underwent embryo transfer (September 2015-May 2016) with EG in comparison with a historically similar patient population who underwent embryo transfer with traditionally employed culture media (Cleavage or Blastocyst Medium, Quinn's Advantage Medium, SAGE) at the same clinic in 2014.

Primary objective
To compare the CPR between the EG transfer patient group and standard transfer patient group.

Secondary objective

To compare the fertilization rate, embryo quality rate [day 2, 3 and 5] and miscarriage rate between EG transfer patient group and standard transfer patient group

- patient demographics (female age, number of oocytes aspirated, embryo quality and number of embryois transferred were included in the analysis,

This comparison will allow it to be determined whether there is a statistically significant improvement in ART outcome, in Drs. Aevitas Fertility Clinic, when the use of EG is implemented as standard protocol. These results will inform future use of HA products to improve patient treatment outcomes in the clinic.

1.6 Hypotheses

Retrospective study

The patients in the PICSI® treatment group are expected to have significantly improved outcomes in terms of fertilisation rate, embryo quality rate [day 2, 3 and 5], OPR and miscarriage rate when compared with the ICSI treatment group using an IVF group as a control.

Prospective study

The implementation of EG used as an embryo transfer medium will result in a significant increase in CPR while reducing miscarriage rate compared to the historically similar group with standard embryo transfer medium.

Chapter 2

Retrospective Study: The Comparison of PICSI ® vs. ICSI on ART outcome

2.1 Materials & Methodology

2.1.1 Study Population

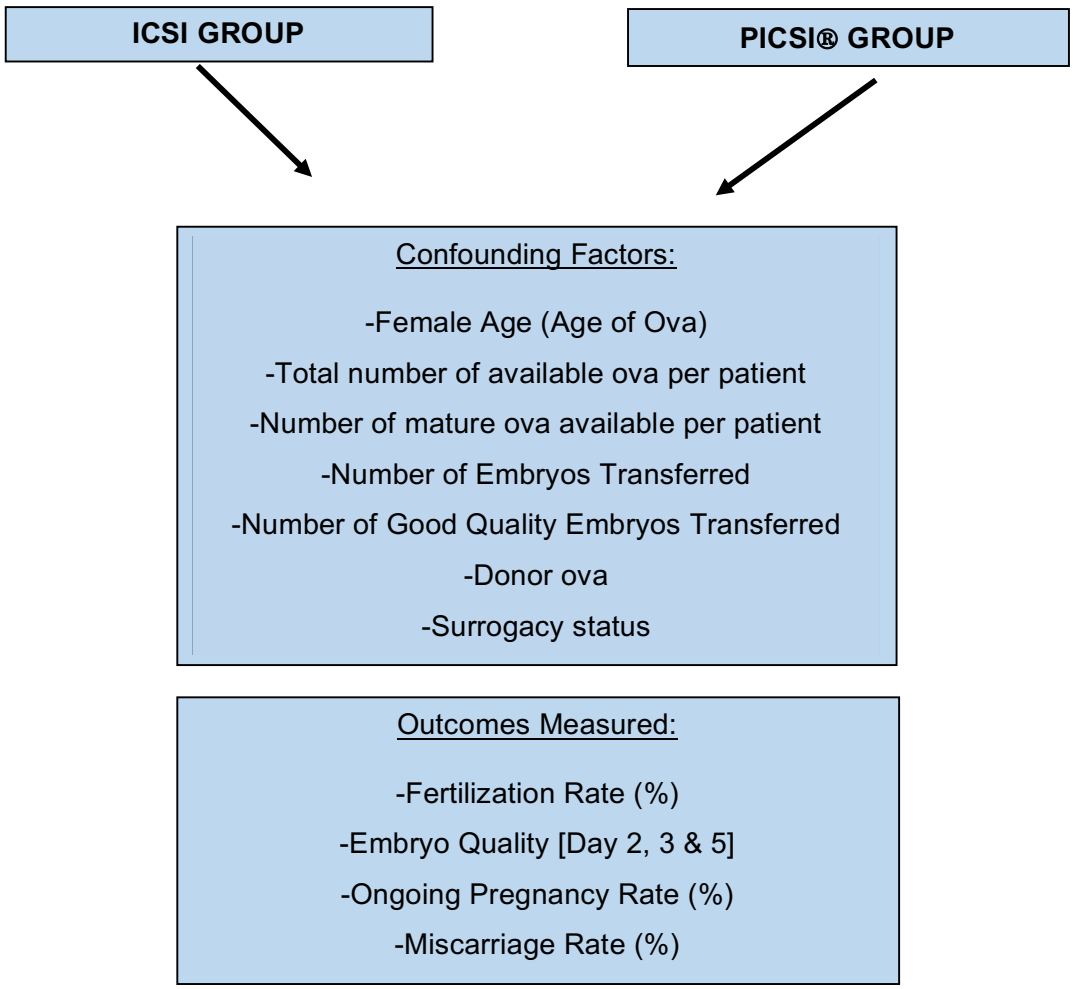
- All PICSI®, ICSI and IVF ART cycles on the clinic spread sheet [2014-2015] – including oocyte donor cycles

    - Exclusion criteria:
        - < 3 MII ova
        - < 3 embryos on day 2
        - No clinical pregnancy result
        - Female age ≥38
        - HIV or Hepatitis B positive patients
        - Frozen Embryo Transfer (FET) cycles
        - Some male factors (Testis Biopsy & OATS)
        - Cycles that made use of more than one fertilization technique

The exclusion criteria were designed to further lessen all possible confounding factors. Patients with less than 3 mature ova retrieved or less than 3 embryos on Day 2 of culture were excluded based on poor prognosis associated with such a low number of available ova. The known effect of advanced maternal age was limited by excluding female patients over age 38. Patients with HIV or Hepatitis B were not included on the basis that their health status may influence their ability to achieve pregnancy. Male patients who underwent testis biopsy to retrieve sperm or were diagnosed with oligoasthenoteratozoospermia (OAT) were excluded from the study to limit the influence of immunological factors and known poor prognosis. Frozen embryo transfer cycles could not be assessed as data regarding the cycle from which the embryos arose was incomplete in terms of embryo quality on specific days evaluated and total number of ova available following aspiration. Patients for whom clinical pregnancy results were unavailable could not be included as this was one of the primary outcomes measured in the study. Patient cycles that made use of more than one fertilization technique were excluded as fertilization method determined the grouping of patients.

2.1.2 Study Design

A retrospective record review:



2.1.3 Study Objectives

Primary objective

To compare the OPR between the PICSI and ICSI groups using the IVF patient group as control.

Secondary objective

To compare the fertilization rate, embryo quality rate [day 2, 3 and 5] and miscarriage rate between the PICSI and ICSI groups using the IVF patient group as control.

- patient demographics (female age, number of oocytes aspirated, embryo quality and number of embryois transferred) were included in the analysis.

2.1.4 Definitions of Pregnancy & Miscarriage

For this retrospective study, pregnancy is presented as ongoing pregnancy (OP) and is defined as the presence of fetal heart with or without final outcome after 20 weeks gestation. Ongoing pregnancy rate [OPR] is expressed per embryo transfer [%].

Miscarriage was definied as the spontaneous loss of a clinical pregnancy that occurs before 20 completed weeks of gestational age (18 weeks post fertilization) (Zegers-Hochschild *et al*, 2009). Miscarriage rate was expressed per transfer [%].

2.1.5 Data Management & Statistical Analysis

All relevant data from eligible PICSI® and ICSI cycles was captured and stored on a spread sheet similar to that of the standard clinic database.

The confounders assessed, to ensure that the ICSI and PICSI® groups were not statistically significantly different, include; age of ova, total number of ova available per patient, number of mature ova available per patient, number of embryos transferred, number of good quality embryos transferred. The effect of donor ova and surrogacy status was also assessed.

The outcomes measured and compared between the two groups included; fertilization rate (%), embryo quality [Day 2, 3 & 5], clinical pregnancy rate (%) and miscarriage rate (%). The IVF group was used as reference unless otherwise indicated.

Data captured included:

1. Ongoing pregnancy rate [OPR]
2. Miscarriage rate
3. Ova age (not female recipient age)
4. Total number of ova retrieved
5. Number of mature (metaphase II) ova retrieved
6. Fertilization rate – ova fertilized/ MII ova retrieved [%]
7. Number of good quality embryos (GQE) on day 2, 3 and 5 respectively
8. Total number of embryos transferred
9. Number of good quality embryos (GQE) transferred
10. Cycle number of treatment

2.1.6 Procedures

Ovarian stimulation

Female partners underwent controlled ovarian hyper-stimulation according to standardized stimulation protocols. The appropriate stimulation protocol is determined based on patient presentation and medical history, namely; diagnosis, physiology, endocrinology and age. Standard stimulation protocols include; i. Short Protocol, ii. Long Protocol, iii. Natural Cycle, iv. Modified Natural Cycle and v. Antagonist Protocol. Stimulation protocol sub-types were designated individualized codes for the purpose of accurate data collection.

Stimulation involves administration of gonadotrophin-releasing hormone agonist (GnRHa) followed by human menopausal gonadotrophins (HMG) and/or pure follicle stimulating hormone (FSH) from cycle day 3. Follow-up procedures included estradiol determinations and ultrasonographical measurements of the Graafian follicle. Once the leading follicle had reached a diameter of 18mm. Human chorionic gonadotropin (HCG) was administered to induce ovulation.

Aspiration and gamete handling

Standard procedures were used [Appendix III & XII].

Semen preparation

Sample characteristics such as sperm count and motility determined the semen preparation method used. Standard wash and swim-up techniques, using HEPES buffered sperm preparation medium, were used to isolate motile spermatozoa during semen preparation when possible. Gradient (90%, 45%) centrifugation method was used for samples with parameters below reference ranges as determined by the World Health Organisation (WHO, 2010). [Appendix XI].

ICSI Method

Refer to Appendix I & II.

PICSI® Method

Refer to Appendix VII.

IMSI Method

Refer to Appendix VIII.

Embryo culture and evaluation

Standard protocol descriptions for Embryo Culture & evaluation can be observed in Appendix III.

Embryo transfer

Standard protocol was used. Embryos selected for transfer were placed in 1ml CO_2 equilibrated, warmed [37°C] culture medium (Cleavage or Blastocyst medium, SAGE) before embryo transfer. Thereafter standard routine embryo transfer protocol was followed. [Appendix XIV]

Embryo Quality

Embryo quality was evaluated according to standard protocol in the clinic based on the work of Veeck, 1999 & Veeck *et al,* 2003. [Appendix IV]. Blastocyst grading is according to a modified Gardner & Schoolcraft grading system [Appendix V].

Embryo Vitrification & Warming

Refer to Appendix XIII.

Pregnancy

Ongoing pregnancy (OP) is defined as the presence of fetal heart with or without final outcome after 20 weeks gestation for the purposes of the study. Ongoing pregnancy rate [OPR] is expressed per embryo transfer [%].

Miscarriage

Miscarriage was definied as the spontaneous loss of a clinical pregnancy that occurs before 20 completed weeks of gestational age (18 weeks post fertilization) (Zegers-Hochschild *et al*, 2009). Miscarriage rate was expressed per transfer [%].

Consent Forms

Waiver of consent was approved by the Health Research Ethics Committee (Ethics Reference #: S15/03/052).

2.2 Results

2.2.1 Patient Population

The patient population included in the retrospective study was comprised of 388 patients. The respective sample sizes of relevant procedure groups were as follows; 41 IVF patients, 184 ICSI patients and 163 PICSI® patients. The exclusion criteria were designed to eliminate variables other than the fertilization method (ICSI or PICSI®) that could potentially have influenced treatment outcomes. The comparison of PICSI® and ICSI treatment groups was the primary focus of this portion of the study. Cycles that made use of more than one fertilization technique, such as those where the number of ova retrieved for a single patient were divided into IVF and ICSI groups, were excluded from the analysis to limit confounding factors. The IVF treatment group was used as the reference group in the regression analyses but the estimated contrast between PICSI® and ICSI treatment groups was the main objective.

2.2.2 Potential Confounders

To ensure an unbiased comparison between the ICSI and PICSI® treatment groups, adjustments were made for confounders. A comparison between the treatment groups with respect to potential confounders are provided in Table 2.1 and graphical presentation using side-by-side boxplots are provided in Figures 3.1 - 3.4.

Table 2.1 Descriptive statistics and comparison of potential confounders for the three treatment groups; PICSI® and ICSI (ref IVF).

Variable	IVF (n=41) Mean (SD)	PICSI® (n=163) Mean (SD)	ICSI (n=184) Mean (SD)	p-value
Age of ova (years)	30.732 (4.64)	30.767 (4.62)	29.913 (5.10)	0.229
Total ova (number)	11.9 (7.3)	12.9 (7.7)	13.7 (7.6)	0.344
Mature ova (number)	10.6 (6.2)	10.9 (6.6)	11.0 (6.2)	0.936
Total Transferred (number)	2.07 (0.57)	1.97 (0.56)	2.14 (SD ± 0.74)	0.0598[1]
GQE Transferred (number)	1.65 (0.70)	1.56 (0.76)	1.62 (SD ± 0.80)	0.536[1]

1: chi-square test

Age of Ova

A box and whisker plot in Figure 2.1 illustrates the mean age of ova, within each of the defined treatment groups. To account for those patients making use of donor ova, the age of ova was used rather than the age of female patient being treated. Each box represents the distribution of all ova ages among patients in respective group; the horizontal line within each box indicates the median or 50th percentile of the given group. The bottom margin of each box further represents the 25th percentile and the top margin represents the 75th percentile. Mean ova ages where: 30.73 (SD ± 4.64) years among IVF patients, 30.77 (SD ± 4.62) years among PICSI® patients and 29.91 (SD ± 5.10) years among ICSI patients (Table 2.1). An analysis of variance (ANOVA), which looks at intergroup and intragroup variability, found no significant difference, as indicated by an adjusted p value of 0.229 (significance is indicated by p= <0.05).

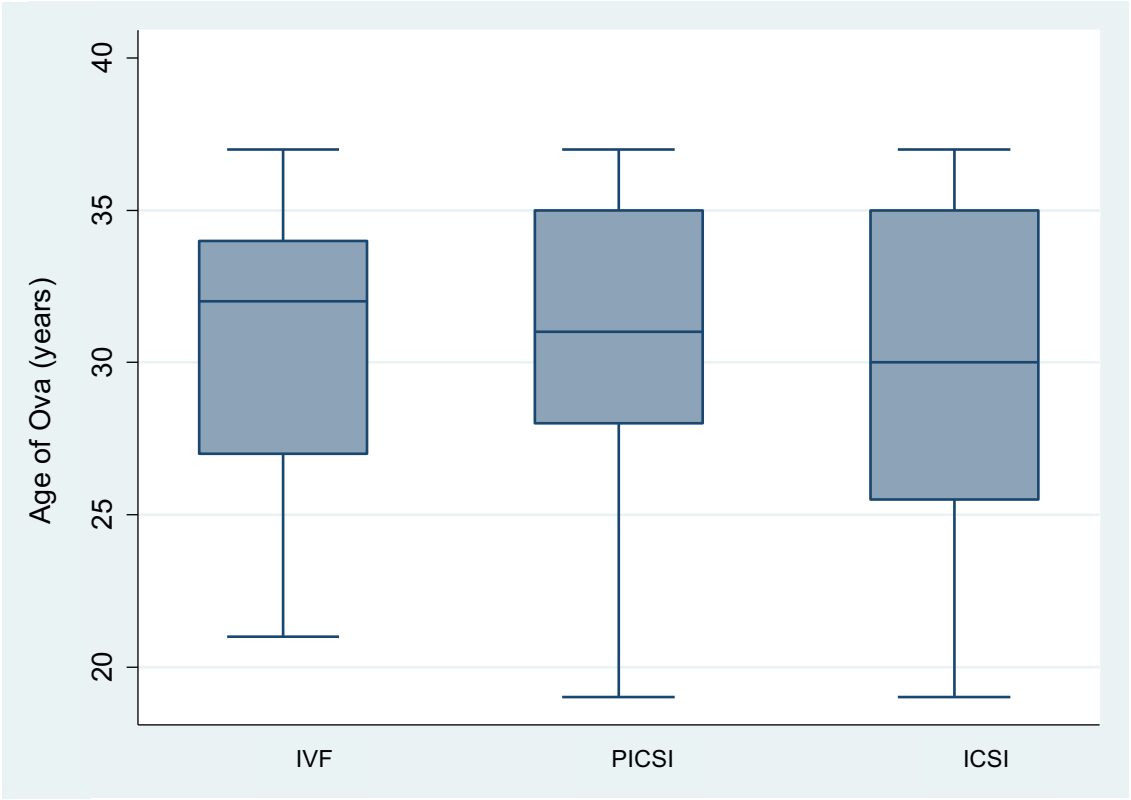


Figure 2.1 A box plot illustrating the distribution of patient ages within respective procedure groups evaluated; PICSI® and ICSI (ref IVF).

Total Number of Ova Per Patient

A box and whisker plot in Figure 2.2 illustrates the mean number of ova retrieved per patient. The number of total ova retrieved per patient was very similar among IVF, ICSI and PICSI® groups. The means are 11.9 (SD ± 7.3) ova per IVF patient, 12.9 (SD ± 7.7) ova per PICSI® patient and 13.7 (SD ± 7.6) ova per ICSI patient (Table 2.1). Points plotted on the graph represent single events that are outlying in this distribution. These events are likely accounted for by patients that experienced ovarian hyperstimulation syndrome (OHSS) or patients with polycystic-ovarian syndrome (PCOS). There was no significant difference as indicated by an adjusted p value of 0.344 (ANOVA).

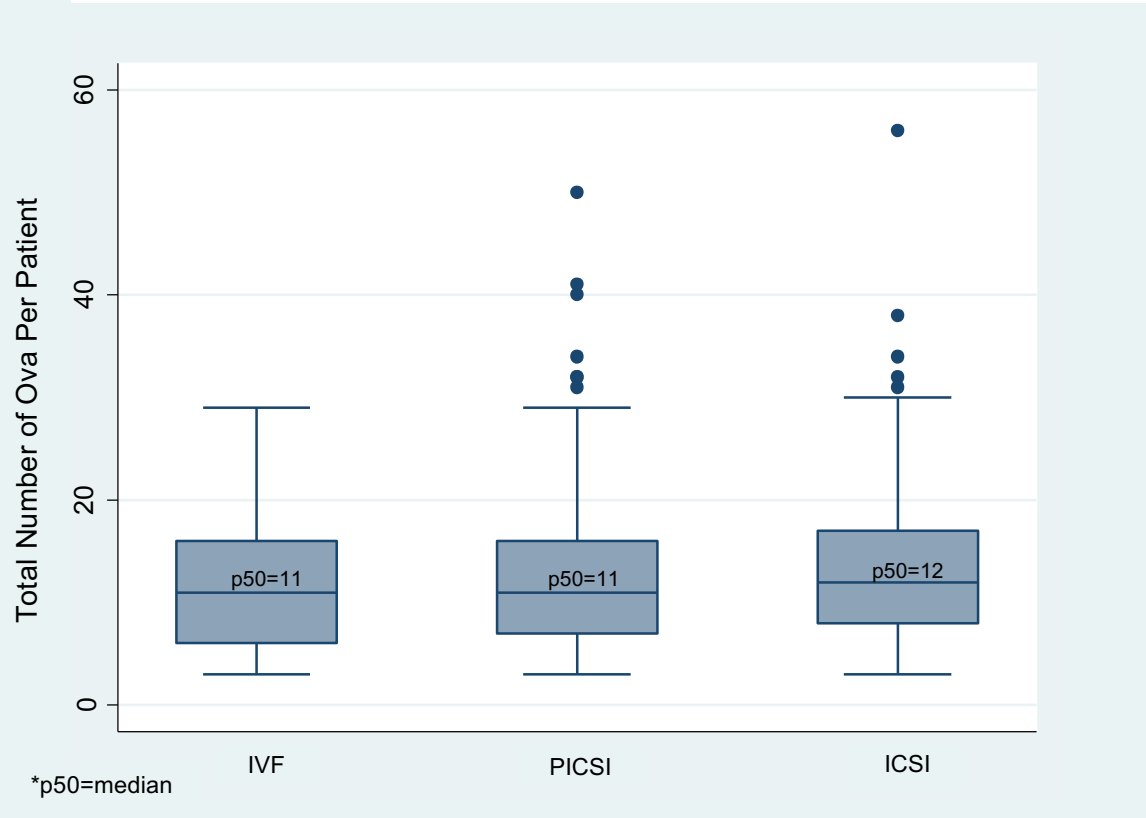


Figure 2.2 A box plot illustrating the total number of ova retrieved per patient within respective treatment groups; PICSI® and ICSI (ref IVF).

Number of Mature [MII] Ova Per Patient

The number of mature, Metaphase II, ova retrieved per patient within each treatment group is represented as a box and whisker plot (Figure 2.3). Mean numbers of mature ova retrieved where: 10.6 (SD ± 6.2) ova per IVF patient, 10.9 (SD ± 6.6) ova per PICSI® patient and 11.0 (SD ± 6.2) ova per ICSI patient (Table 2.1). The mean number of mature ova retrieved per patient among treatment groups and found no significant difference, as indicated by an adjusted p-value of 0.936 (ANOVA).

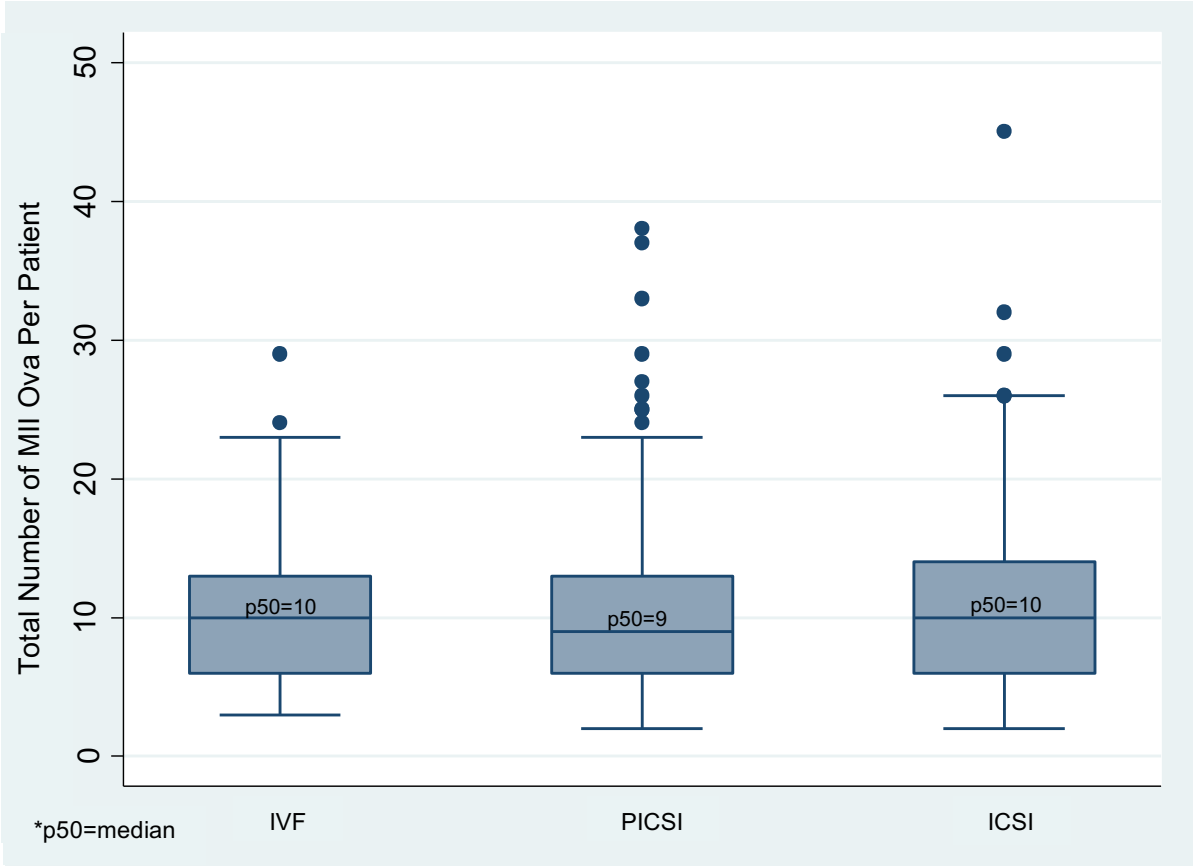


Figure 2.3 A box plot illustrating the number of mature, Metaphase II, ova retrieved per patient within respective treatment groups; PICSI® and ICSI (ref IVF).

Embryos Transferred

The total number of embryos transferred per patient for IVF, PICSI® and ICSI treatment groups respectively is represented in Table 2.2. The mean number of GQE transferred is two in all three procedure groups (Figure 2.4). A chi-square test comparing proportional profile between treatment groups was not significant (p=0.0598).

Table 2.2 Number of embryos transferred per patient in respective groups; PICSI® and ICSI (ref IVF).

Number Transferred	IVF	PICSI®	ICSI
1	2 (4.88%)	10 (6.13%)	6 (3.26%)
2	31 (75.61%)	130 (79.75%)	137 (74.46%)
3	7 (17.07%)	17 (10.43%)	32 (17.39%)
4	0 (0.00%)	0 (0.00%)	0 (0.00%)
5	0 (0.00%)	0 (0.00%)	1 (0.54%)
6	0 (0.00%)	0 (0.00%)	2 (1.09%)
Total:	41 (100.00%)	163 (100.00%)	184 (100.00%)

Patients in the ICSI group tended to have more embryos transferred compared to patients in the PICSI® treatment group. In the ICSI group, 19% of patients had more than two embryos transferred whereas only 10% of PICSI® patients had more than 2 transferred (Table 3.2). A Fishers exact confirmed no significant overall difference in group profiles (p=0.536)

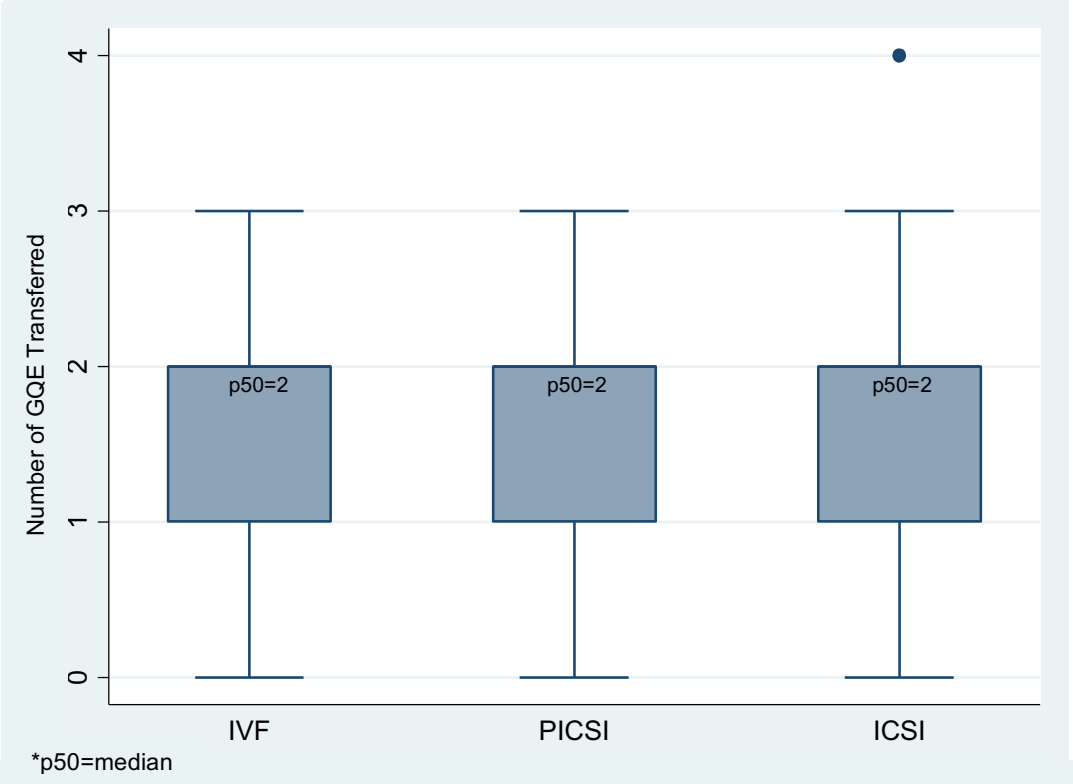


Figure 2.4 A box plot illustrating the number of GQE transferred per patient within respective treatment groups; PICSI® and ICSI (ref IVF).

2.2.3 Measured Outcomes

Based on the hypothesis, patients in the PICSI® treatment group were expected to have better outcomes in terms of fertilisation rate, embryo quality rate [day 2, 3 and 5], ongoing clinical pregnancy rate, and miscarriage rate when compared with the ICSI treatment group. The measured outcomes summarized in Table 2.3 show no statistically significant differences between PICSI® and ICSI treatment groups. The absolute number of mature ova fertilized per patient was comparable in PICSI® and ICSI groups, as well as the fertilization rate. The estimated median difference in fertilization rate between ICSI and PICSI® is 3.1% (95% CI: -2.0 to 8.3%), p=0.226. Although not statistically significant, PICSI® or ICSI fertilization technique did have a marginal effect on the rate of successful fertilization. The differences in the proportion of good quality embryos resulting from PICSI® and ICSI were not statistically significant on embryo culture days 2, 3 or 5. In testing for overall procedure effect without using IVF as reference, the calculated risk difference of PICSI® compared with ICSI was 7.6% (95% CI: -3.1 to 18.3%). A 7.6% lower clinical pregnancy rate in the PICSI® treatment group, was found not to be significant (p=0.164). Finally, a Fishers exact indicated no statistical significant difference in the miscarriage rates among ICSI and PICSI® treatment groups (p=0.779).

Table 2.3 Comparison of the measured outcomes for the respective groups; PICSI® and ICSI (ref IVF).

Variable	IVF (n=41) Mean (SD)	PICSI® (n=163) Mean (SD)	ICSI (n=184) Mean (SD)	p-value
Number ova Fertilized	8.6 (5.1)	8.3 (5.0)	8.8 (5.1)	0.6853
Fertilization Rates (%)	0.837 (0.2)	0.774 (0.2)	0.804 (0.2)	0.0799
Embryo Quality Day 2 (%)	0.65 (0.2)	0.54 (0.2)	0.56 (0.2)	0.318
Embryo Quality Day 3 (%)	0.46 (0.2)	0.39 (0.2)	0.40 (0.2)	0.597
Embryo Quality Day 5 (%)	0.24 (0.2)	0.21 (0.2)	0.23 (0.2)	0.120
OPR (%)	53.66% [22/41]	40.40% [61/151]	48.33% [87/180]	0.196
Miscarriage Rate (%)	2.44% [1/41]	5.52% [9/163]	4.35% [8/184]	0.779

Number Fertilized

The number of mature, Metaphase II, ova that successfully fertilized per patient is represented as a box and whisker plot in Figure 2.5. Mean numbers of mature ova fertilized were 8.6 (SD ± 5.1) ova per IVF patient, 8.3 (SD ± 5.0) ova per PICSI® patient and 8.8 (SD ± 5.1) ova per ICSI patient (Table 2.3). The mean number of mature ova fertilized per patient among treatment groups was compared by ANOVA and there was no significant difference (p= 0.6853).

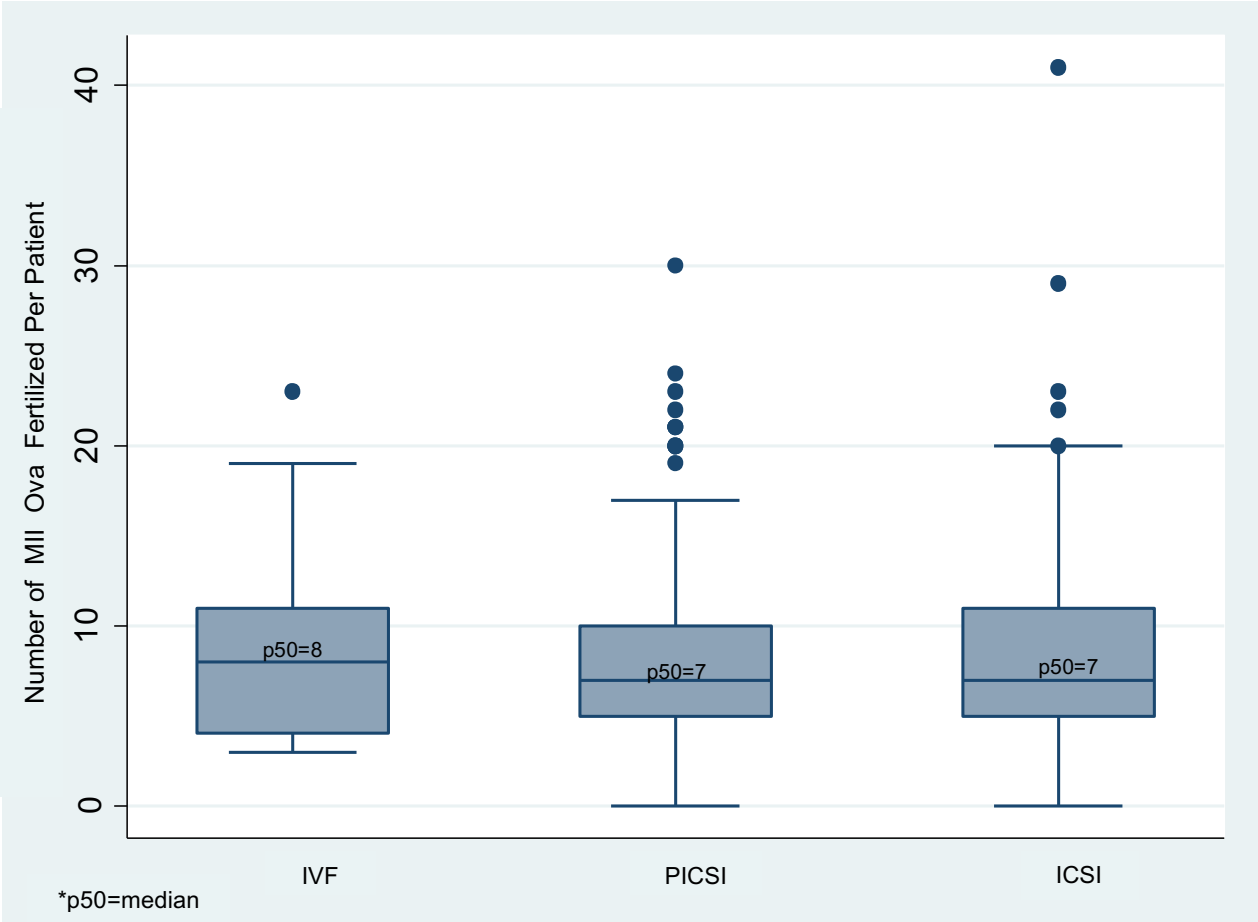


Figure 2.5 A box plot illustrating the number of mature ova fertilized per patient within respective treatment groups; PICSI® and ICSI (ref IVF).

Fertilization Rate

The fertilization rates of available MII ova were 83.7% [201/240] in the IVF group, 77.4% [1298/1677] in the PICSI® group and 80.4% [1211/1506] in the ICSI group (Table 2.3). The points plotted below the whiskers represent cases of complete fertilization failure (Figure 2.6). The mean fertilization rates of MII ova among treatment groups was compared by ANOVA and there was no significant difference (p=0.0799).

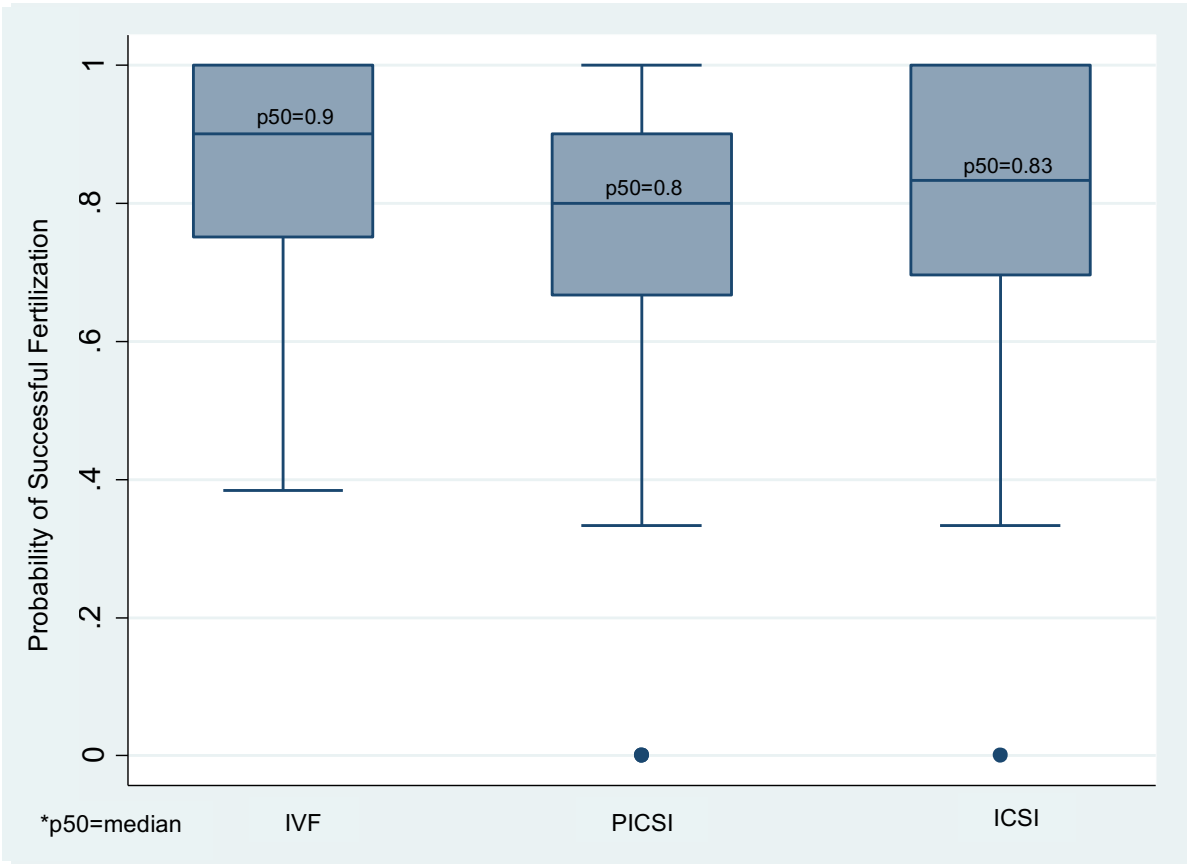


Figure 2.6 A box plot illustrating the probability, between 0 and 1, of successful fertilization occurring in available MII ova among respective treatment groups; PICSI® and ICSI (ref IVF).

A quantile non-parametric regression model was used to model the outcome fertilization rates in MII ova on procedure group adjusting for a number of covariates; age of ova, donor and surrogate status. The adjustments for relevant covariates were made to take into account possible difference in the treatment groups due to a non-randomised study design. The study was retrospective observational and therefore randomization was not possible. The model indicated that neither donor ova status (p = 0.485) nor surrogacy status (p = 0.552) were related to the observed fertilization rates. When testing for overall procedure effect the p value

of 0.0811 indicated no group effect. Estimating the specific contrasts, the estimated median difference in fertilization between IVF and PICSI® is 9.2% (95% CI: 0.9% to 17.5%) with PICSI® lower than IVF. A p value of 0.029 confirms that the difference is significant. The estimated median difference in fertilization between IVF and ICSI of 6.1% (95% CI: -2.2% to 14.3%) with ICSI lower than IVF, was not significant (p=0.148). The estimated median difference in fertilization rate between ICSI and PICSI® is 3.1% (95% CI: -2.0 to 8.3%), p=0.226. There was no significant difference in fertilization rate of MII ova between PICSI® and ICSI techniques.

Embryo Quality Rate

The box and whisker plot (Figure 2.7) based on raw data contains descriptive statistics that serves to illustrate the tendency of a decline in number of good quality embryos during the culture period regardless of fertilization technique employed. Points plotted beyond the whiskers of the box plot represent cases with a large number of ova available at the given time point.

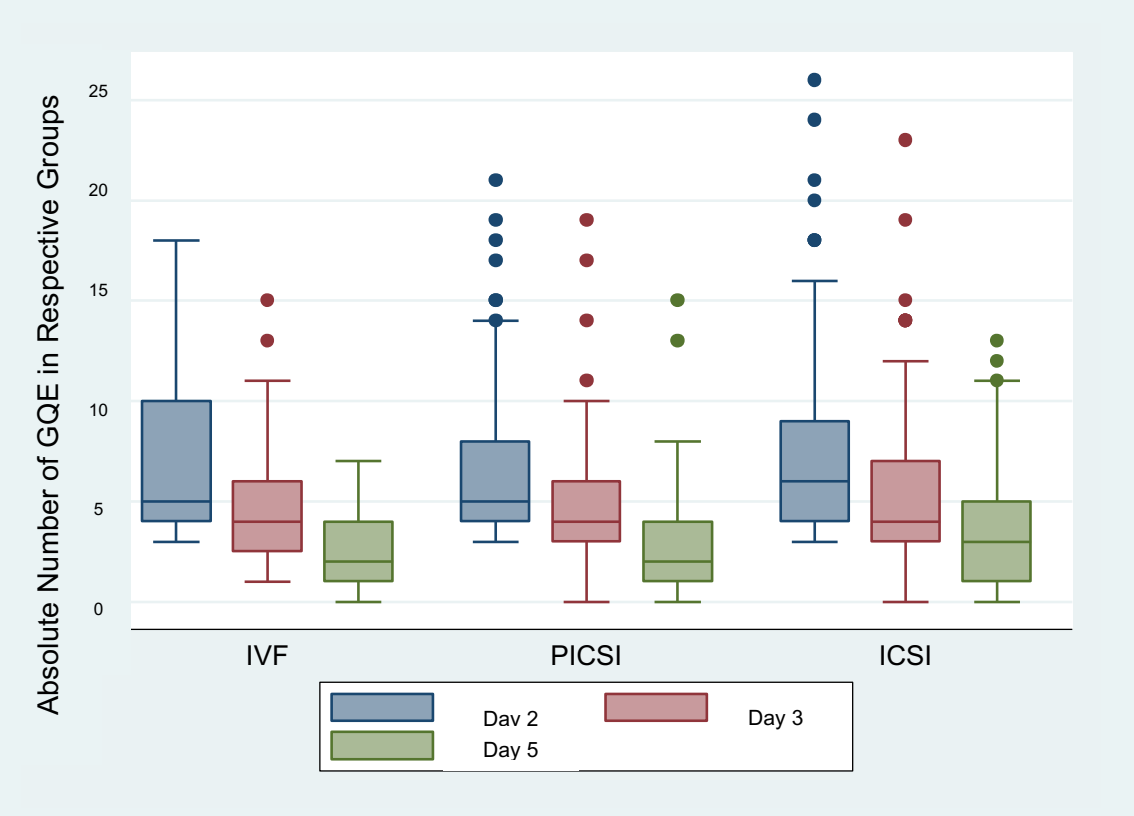


Figure 2.7 A box plot illustrating the absolute number of GQE, based on specified criteria, on embryo culture days 2, 3 and 5 within each treatment group; PICSI® and ICSI (ref IVF).

The proportion of good quality embryos relative to the number of available ova retrieved per patient is highest in the IVF treatment group, as is expected given it has the highest fertilization rate among the treatment groups. In the PICSI® treatment group, the proportion of good quality embryos resulting relative to the total number of available oocytes after aspiration is approximately 54% [535/991] on day 2, 39% [258/661] on day 3 and 21% [58/276] on day 5 (Table 2.3). In comparison, 56% [469/837] of the available oocytes in the ICSI treatment group resulted in good quality embryos on day 2, 40% [216/539] good quality on day 3 and 23%

[51/219] good quality on day 5. The profiles of ICSI and PICSI® are nearly identical across all days evaluated and very similar to the IVF control group (Figure 2.8).

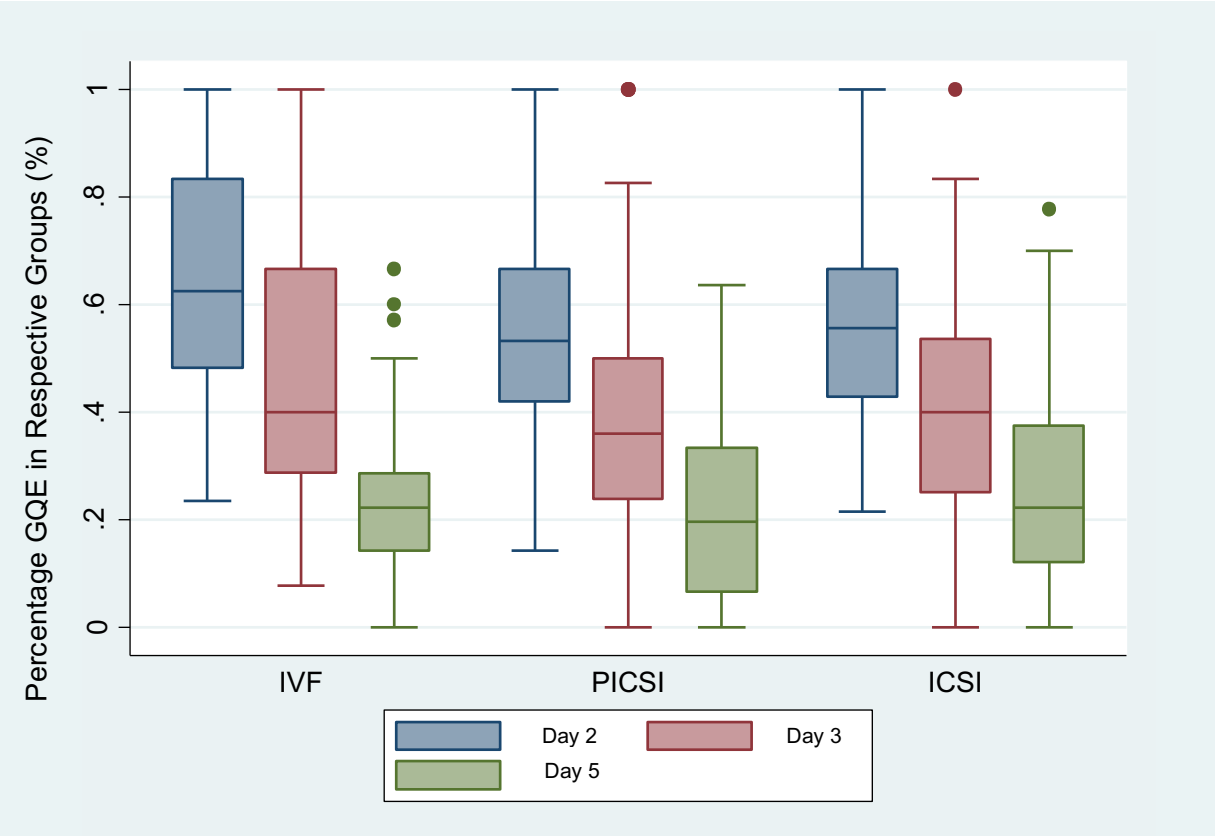


Figure 2.8 A box plot illustrating the percentage of GQE at each time point for respective treatment groups; (PICSI® and ICSI (ref IVF)) over all embryo culture days of study (Day 2,3 & 5).

A two-sample t test, with equal variances, compared the proportions of good quality embryos on Day 2, 3 and 5 per patient (Table 2.4) separately for the ICSI and PICSI® treatment groups. The approximate difference in the proportion of good quality embryos between the PICSI® and ICSI treatment groups was 1.97% on **Day 2**, 1.17% on **Day 3** and 3.36% on **Day 5.** The differences in the proportion of good quality embryos resulting from PICSI® and ICSI on embryo culture day 2 (p=0.318), day 3 (p=0.597) and day 5 (p=0.120) were not statistically significant.

Table 2.4 Comparison of GQE rate between PICSI® and ICSI groups.

Day	Sample Size	PICSI®	Sample Size	ICSI	Mean Difference	95% Confidence Intervals	p-value
2	160	0.54	177	0.56	-0.02	-.06 to.02	0.318
3	160	0.39	176	0.40	-0.01	-.06 to .03	0.597
5	120	0.21	135	0.25	-0.03	-.8 to .01	0.120

Ongoing Pregnancy Rates (OPR)

The OPR were 53.66% [22/41] in the IVF treatment group, 48.33% [87/180] in the ICSI treatment group and 40.40% [61/151] in the PICSI® treatment group and the intergroup differences observed were not significant (p=0.196) (Table 2.3). These OPR are represented as a bar chart in Figure 2.9.

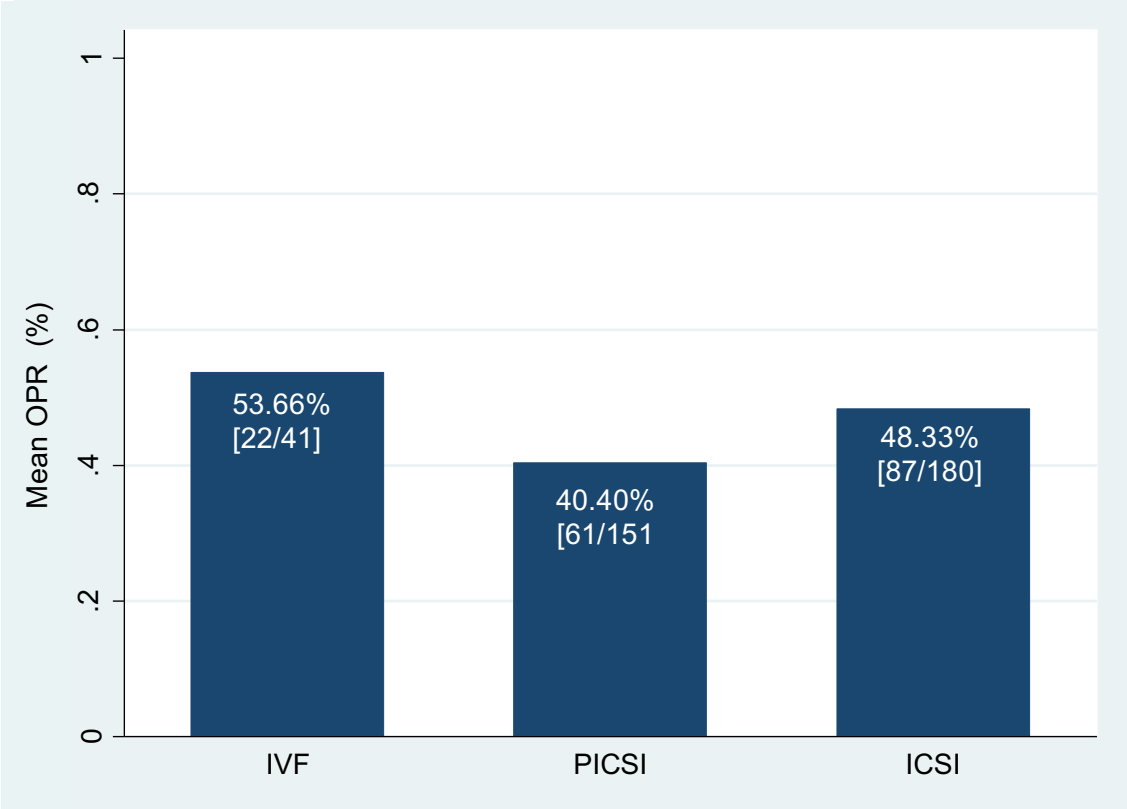


Figure 2.9 A bar chart showing the OPR of the three respective treatment groups; PICSI® and ICSI (ref IVF).

A binomial model of pregnancy outcome on procedure adjusted for age of ova, fertilization method (IVF, PICSI® or ICSI) donor status, number of mature ova and surrogacy status to account for the selection bias of patient grouping to allow a fair comparison of the overall effect of fertilization method on pregnancy outcome. The calculated risk difference for PICSI® compared to IVF was 15.3% (Risk Difference= PICSI®/IVF) and a p value of 0.080 indicated no statistically significant difference. Similarly, the risk difference for ICSI compared to IVF was 7.7% (Risk Difference= ICSI/IVF) with a p value of 0.373 also not statistically significant. The risk difference calculated based on donor status was 19.5% (Risk Difference: No Donor Ova/Donor Ova] indicating a significantly higher OPR in those patients who did make use of donor ova (p=0.016). The calculated risk difference for surrogacy status was 3.1% (Risk

Difference=Surrogate Not Used/ Surrogate Used) was not significant (p=0.849). In terms of the number of available mature ova per patient the risk ratio was 0.7% p=0.064.

In testing for overall procedure effect without using IVF as reference, the calculated risk difference of PICSI® compared with ICSI was 7.6% (95% CI: -3.1 to 18.3%). A 7.6% lower OPR in the PICSI® treatment group, was not significant (p=0.164).

Miscarriage Rates

The miscarriage rate for the IVF treatment group was 2.44% [1/41], 4.35% [8/184] in the ICSI treatment group and 5.52% [9/163] in the PICSI® treatment group (Table 2.3 & Figure 2.10). The study used a unified definition of pregnancy loss for all types of miscarriages, at various stages of pregnancy, to form a single binary outcome. A Fishers exact indicated no statistical significant difference in the miscarriage rates among IVF, ICSI and PICSI® treatment groups (p=0.779).

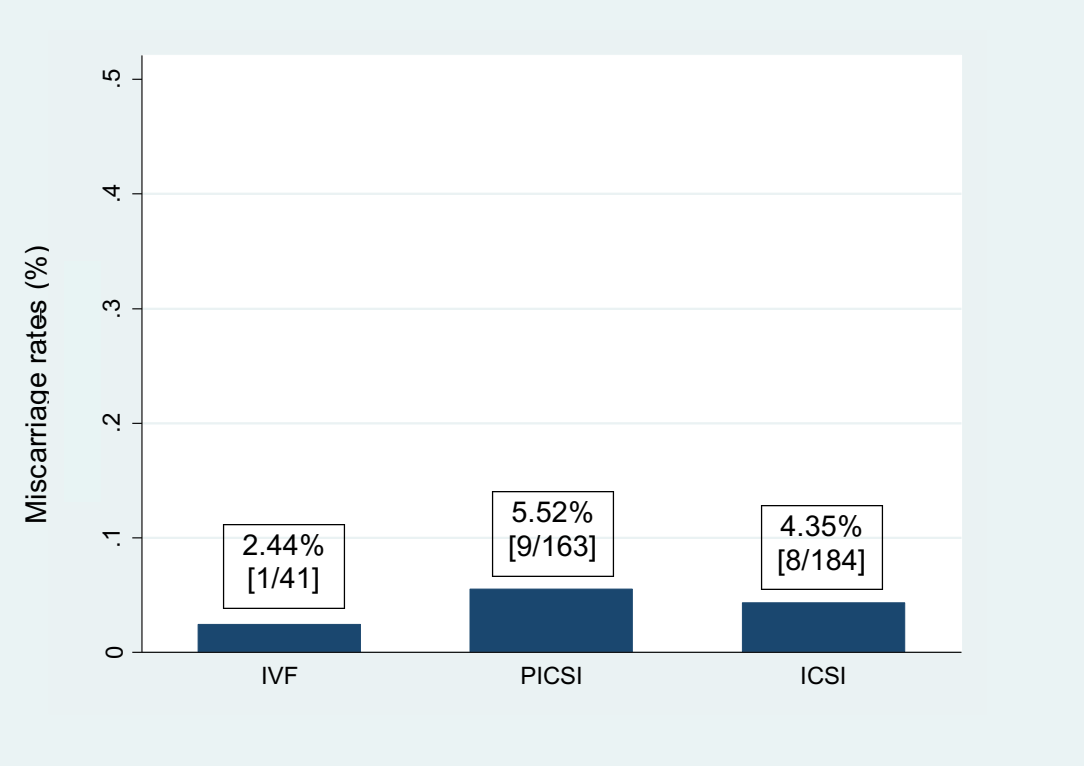


Figure 2.10 A bar chart showing the miscarriage rates for each of the three respective treatment groups; PICSI® and ICSI (ref IVF).

2.3 Discussion

The study found no significance in terms of fertilization rate, number of good quality embryos on day 2, 3, or 5 nor in OPR or miscarriage rates between PICSI® and ICSI. Although not statistically significant, ICSI outcomes were consistently superior compared with PICSI® in all measured outcomes.

Confounding Factors

To ensure that the ICSI and PICSI® groups could be compared statistically, the following potential confounding factors were assessed with the appropriate statistical tests; age of ova, total number of ova, number of mature ova available per patient, total number of embryos transferred and number of good quality embryos transferred. The effect of donor ova use and surrogacy status were also assessed. Statistical analyses with p-values of <0.05 were considered statistically significant. When interpreting statistical findings, it is important to note that the failure to detect statistically significant differences does not simply imply equivalence. An inadequate sample size can result in the failure to detect a difference (Cleophas & Zwinderman, 2012). In other instances, although detected, a statistically significant difference may not have clinical significance. We are confident that the reported outcomes were mainly due to fertilization (ICSI and PICSI®) technique.

The factors identified and analysed as potential confounders were based on relevant literature. Age of ova (female age) was identified as a potential confounder based on the vast body of literature that exists to demonstrate an age-dependent decline in female fertility (Steiner & Jukic, 2016). The following age-related abnormalities affect the developmental competence of oocytes. Meiotic incompetence or inability to complete meiotic maturation could result in oocytes incapable of fertilization. Errors in meiosis that are compatible with fertilization may lead to compromised embryo viability and genetic abnormalities. Cytoplasmic deficiencies, expressed in various stages before or after fertilization, also become more common with advancing age (Armstrong, 2001). Maternal aging-associated oocyte aneuploidy is equally well documented (Kuliev *et al*, 2003). Oocyte donation has the potential to extend the reproductive potential in women of advanced reproductive age. Studies have reported improved outcomes with regard to fertilization rates, number of embryos transferred, embryo implantation rate and clinical pregnancy rates in women below 40 years of age (Sauer *et al,* 1990 & 1992). Donor status therefore also needed to be accounted for in statistical workings for patients making use of oocyte donation due to premature ovarian failure or advanced maternal age. In the current study oocyte age was not significantly different between the ICSI and PICSI® groups (30.8 and 29.9 years respectively) and had therefore no impact on the pregnancy or miscarriage rates.

The total number of available ova per patient was identified as a potential confounder as studies have documented the association between number of eggs and live birth rate in IVF treatment. All IVF cycles performed in the UK from 1991 to 2008 were analysed to produce a nomogram to predict the live birth rate following IVF based on the number of eggs and the age of the female. The number of eggs shown to maximize live birth rate is 15 (Sunkara *et al*, 2011). The number of mature ova available per patient is also a predictor of outcome and although immature oocytes retrieved have the potential to mature *in vitro* and to fertilize normally, studies have illustrated that resultant embryos are of poorer quality and have reduced implantation potential compared to their mature counterparts (Reichman *et al*, 2010). Embryos derived from *in vitro* matured oocytes have also been observed to have particularly high aneuploidy rates, mainly complex mosaics (Strassburger *et al*, 2010). In the current study, the total number of ova and the number of mature ova were not significantly different between the ICSI and PICSI® groups (13.7 vs. 12.9 and 11.0 vs. 10.9 respectively) and therefore had no impact on the pregnancy or miscarriage rates.

The total number of embryos transferred was assessed based on findings that elective single embryo transfers result in a lower pregnancy rate than a double embryo transfer in a fresh cycle (Martikainen *et al*, 2001). Based on a systematic review of the Cochrane Central Register of Controlled Trials, elective single embryo transfers are however associated with a higher chance of delivering a term singleton live birth compared with double embryo transfer (McLernon *et al*, 2010). The multiple pregnancy rate after elective single embryos transfers is comparable with that observed in spontaneous pregnancies and is an important consideration to support efforts in reducing dizygotic twin rates in ART (Thurin *et al*, 2004). In the current study, the number of embryos transferred was not significantly different between the ICSI and PICSI® groups (2.14 vs. 1.97) and had therefore no impact on the pregnancy or miscarriage rates.

The quality of embryos transferred was assessed, as blastocyst grading remains the greatest predictor of developmental potential, successful implantation and embryo transfer outcome (Balaban *et al*, 2000). The graduated embryo score predicts blastocyst formation and pregnancy rate from cleavage-stage embryos (Fisch *et al*, 2001) and transfer of good quality embryos results in increased pregnancy rates (Rienzi *et al,* 2005). In the current study, the number of good quality embryos transferred was also not significantly different between the ICSI and PICSI® groups (1.62 vs. 1.56) and had therefore no impact on the pregnancy or miscarriage rates.

Benefit of PICSI® for Sperm Selection

For sperm used in ICSI fertilization, current sperm selection techniques depend solely on sedimentation (gradient centrifugation) or migration of the sperm (swim-up) in combination with morphological assessment. Important characteristics such as DNA integrity, apoptosis, membrane maturation and ultrastructure of the spermatozoa are not considered (Said & Land, 2011). The use of specialized PICSI® culture during the fertilization of oocytes provides an additional parameter for the selection of normal spermatozoa. The binding of spermatozoa to the hyaluronic acid microdots on the bottom surface of the dish serve as a biochemical marker to identify functionally and developmentally mature spermatozoa for injection (Huszar *et al.,* 2006. 2007, 2012). Spermatozoa able to bind to the hyaluronic acid have a chromatin structure with high DNA chain integrity associated with fertilization competence and normal chromosomal constitution (Yagni *et* al, 2010). The use of PICSI® dish spermatozoa culture during the fertilization of oocytes provides an objective parameter in the evaluation and selection of spermatozoa for injection, minimizing inter- and intra-individual variation. The operator must still assess the morphological features of the bound spermatozoa to select a single spermatozoon for injection. Use of the PICSI® technique is not appropriate for patients with below normal sperm concentration and/or motility as the effectiveness of the dish depends on the mobility of spermatozoa to reach and bind to the microdots.

Comparison of Results with the Relevant Literature

PICSI® and Fertilization Rate

The results of the current study showed no significant improvement in fertilization rate for PICSI® over ICSI (77.4% vs 80.4%, respectively).

The literature reporting on fertilization and PICSI® is contradictory. According to research, spermatozoa able to bind to solid-state hyaluronic acid have a chromatin structure with high DNA chain integrity associated with fertilization competence and normal chromosomal constitution (Yagni *et al*, 2010). However, a 2014 Cochrane review reported no statistically significant difference in fertilization rate for PICSI® over other fertilization methods (McDowell *et al*, 2014). A recent 2016 meta-analysis was published by Beck-Fruchter *et al.* This systematic review and meta-analysis searched *PubMed* and *Cochrane* for studies, published up to June 2015, describing the clinical outcome of ICSI cycles in which spermatozoa were selected based on their ability to bind to hyaluronic acid (Beck-Fruchter *et al*, 2016). The only restriction applied to the search was species- humans. Only full-text articles, with control group and specified sperm selection technique were included. The Downs and Black Checklist

(Downs & Black, 1998) was used to assess the quality of the seven studies included and meta-analysis was done where data was available (Ciray *et al*, 2008, Van Den Bergh *et al*, 2009, Parmegiani *et al*, 2010a, Parmegiani *et al, 2010b*, Choe *et al*, 2012, Majumdar & Majumdar, 2013, and Worrilow *et al*, 2013). Pooling of all data also indicated no association between hyaluronic acid based sperm selection technique and fertilization rate. The fertilization rate could be calculated and compared in seven studies, with more than 9700 injected oocytes in total. Subgroup meta-analysis of the prospective studies also indicated no improvement in fertilization rate where hyaluronic acid binding was used to select sperm. The results of the current study therefore agree with the literature.

PICSI® and Embryo Quality

The results of the current study showed no significant improvement in embryo quality (culture day 2, 3 and 5) for PICSI® over ICSI (day 2: 54% vs. 56%, day 3: 39% vs. 40% and day 5: 21% vs. 23%, respectively).

Once again, the data from the literature is controversial. A 2010 study concluded that the selection of spermatozoa using the PICSI® method leads to a higher number of spermatozoa without DNA fragmentation and resulted in a relative improvement of resultant embryo quality (Parmegiani *et al*, 2010a). The design of the study included three prospective studies where the main measured outcomes included; assessments of the DNA fragmentation rate and nuclear morphology of HA bound spermatozoa in comparison with non-bound spermatozoa. The authors reported reduced DNA fragmentation and improved nucleus normalcy compared with spermatozoa immersed in PVP. The injection of bound spermatozoa also significantly improved both the development and quality of embryos in a randomized study (Parmegiani *et al*, 2010b). The meta-analysis by Beck-Fruchter *et al*, (2016) also reported that embryo quality, provided in only two studies from the same research group, demonstrated an improvement in the hyaluronic acid binding group. Secondary outcomes measured included cleavage rate, which showed a significant difference in favour of conventional ICSI.

The 2014 Cochrane review included the evaluation of two randomized controlled trials - a total of 581 patients - and concluded however that there was no statistically significant difference reported between groups in terms of embryo quality (McDowell et al, 2014), and is in agreement with the current study.

PICSI® and OPR

The results of the current study showed no significant improvement in OPR for PICSI® over ICSI (40.40% vs. 48.33%, respectively).

A 2010 retrospective record review of 293 couples treated with PICSI® versus 86 couples treated with conventional ICSI concluded that the injection of HA-bound spermatozoa significantly improved both implantation rates (17.1% vs. 10.3%) and embryo quality (Parmegiani *et* al, 2010b).

The concluding remarks for the Cochrane review (McDowell *et al*, 2014) stated that current evidence was insufficient to permit the evaluation of advanced sperm selection strategy effectiveness in ART. No statistically significant difference was reported between groups in terms of pregnancy rate, implantation rate and live birth rate. In the Beck-Fruchter *et al.* meta-analysis study (2016), CPR was calculated per cycle started and included nearly 1300 ART cycles. Meta-analytic pooling of data again revealed no association between PICSI® sperm selection technique and pregnancy rate. Live birth rate was reported for three studies and meta-analysis showed no significant difference between the two groups. These results from the majority of the literature is in agreement with the current study.

PICSI® and Miscarriage rate

The results of the current study showed no significant difference in miscarriage rate between PICSI® and ICSI cycles (5.52% vs 4,35%, respectively).
The data from the literature support this outcome since spontaneous miscarriage rates were comparable or not significantly different in conventional ICSI and PICSI® groups (McDowell *et al.*, 2014; Beck-Fruchter *et al*., 2016).

Limitations of the study

The results differ from the hypothesis in that a statistically significant improvement in pregnancy rates with PICSI® compared with conventional ICSI fertilization method was expected. These results may be due to small sample sizes in both the PICSI® (163 patients) and ICSI (184 patients) treatment groups, although well matched. A retrospective study has a number of disadvantages. When stratifying from an endpoint, one cannot determine causation; only identify association. The study is subject to confounding and even when adjusted for, there may be other risk factors present that were not identified or measured. Sample size also affects the robustness of study findings, and although the study was adequately powered, larger sample

sizes would have optimized study design (Hess, 2004). Female and male diagnosis could not be assessed as potential confounders based on the way in which data is captured in the clinic. Diagnostic factors (etiology of infertility) such as endometriosis could therefore not be adjusted for. Endometriosis, even at mild stage, may have a direct negative effect on oocyte development, embryogenesis and patients with severe endometriosis have a statistically significant lower pregnancy rate and implantation rate than their non-affected counterparts (Barnhart *et al*, 2002). The mechanisms by which endometriosis impairs fertility are varied but proposed dynamics include alteration in inflammatory response, autoimmune factors and local paracrine action of cytokines (Barnhart *et al*, 2002). Conditions such as hydrosalpinges (Vandromme *et al*, 1995 & Chu *et al*, 2015) or fibroids (Somigliana *et al*, 2011) are also associated with poorer outcomes. The availability of data in any retrospective study relies on the collection of data by other persons, for purposes other than research. The specific indication for ICSI treatment at the particular private clinic is unknown and cancelled cycles or freeze-all cycles were not included in the data captured. It follows therefore that the completeness and accuracy of data available for analysis is a factor to consider in any a retrospective study. The numbers analysed were significantly reduced due to the use of exclusion criteria to lessen all possible confounding. Subgroup analysis could not be completed due to small resulting sample sizes from which significance could not be determined.

Conclusion & Recommendations

Based on the results of the present study and a comprehensive literature review, routine application of PICSI® dishes in all ICSI cycles does not result in improved outcomes in terms of fertilization, embryo quality, clinical pregnancy rate or reduced miscarriage rate.

Although hyaluronic acid is a plausible candidate for improved sperm selection based on its *in vivo* role as a natural selector, routine application of PICSI® technique in all ICSI cycles should be considered carefully until the identification of patients that may benefit from PICSI® technique is investigated further. In order to improve cost effectiveness, only patients who have had previously failed ICSI cycles, have had complete fertilization failure with conventional ICSI, arrested embryo development, have known sperm DNA integrity abnormalities and ICSI patients with low hyaluronan binding score (HBA score) should be considered for PICSI® treatment. Further research should include a sample size large enough to detect differences in subgroups based on potential confounders, randomization should be considered and measured outcomes should include live birth rate.

Chapter 3

Prospective Study: The Effect of EmbryoGlue [EG] On ART Outcome

3.1 Materials & Methodology

3.1.1 Study Population

- All consenting couples undergoing ART treatment at the Drs Aevitas Fertility Clinic [September 2015-May 2016] who had a transfer with EG as an embryo transfer medium – **EG Treatment group.**

- A historically similar group of couples at the Drs Aevitas Fertility Clinic [January – December 2014] who had transfer with standard medium (Cleavage or Blastocyst Medium, Quinns Advantage, SAGE) as an embryo transfer medium - **Control group.**

    - The following exclusion criteria were designed to further lessen all possible confounding:

        - No clinical pregnancy result
        - HIV or Hepatitis B positive patients
        - No consent
        - No oocytes and/or embryo transfer
        - Time in EG <10minutes prior to transfer
        - No EG used in treatment group

Donor oocyte cycles and FET cycles were included in the study.

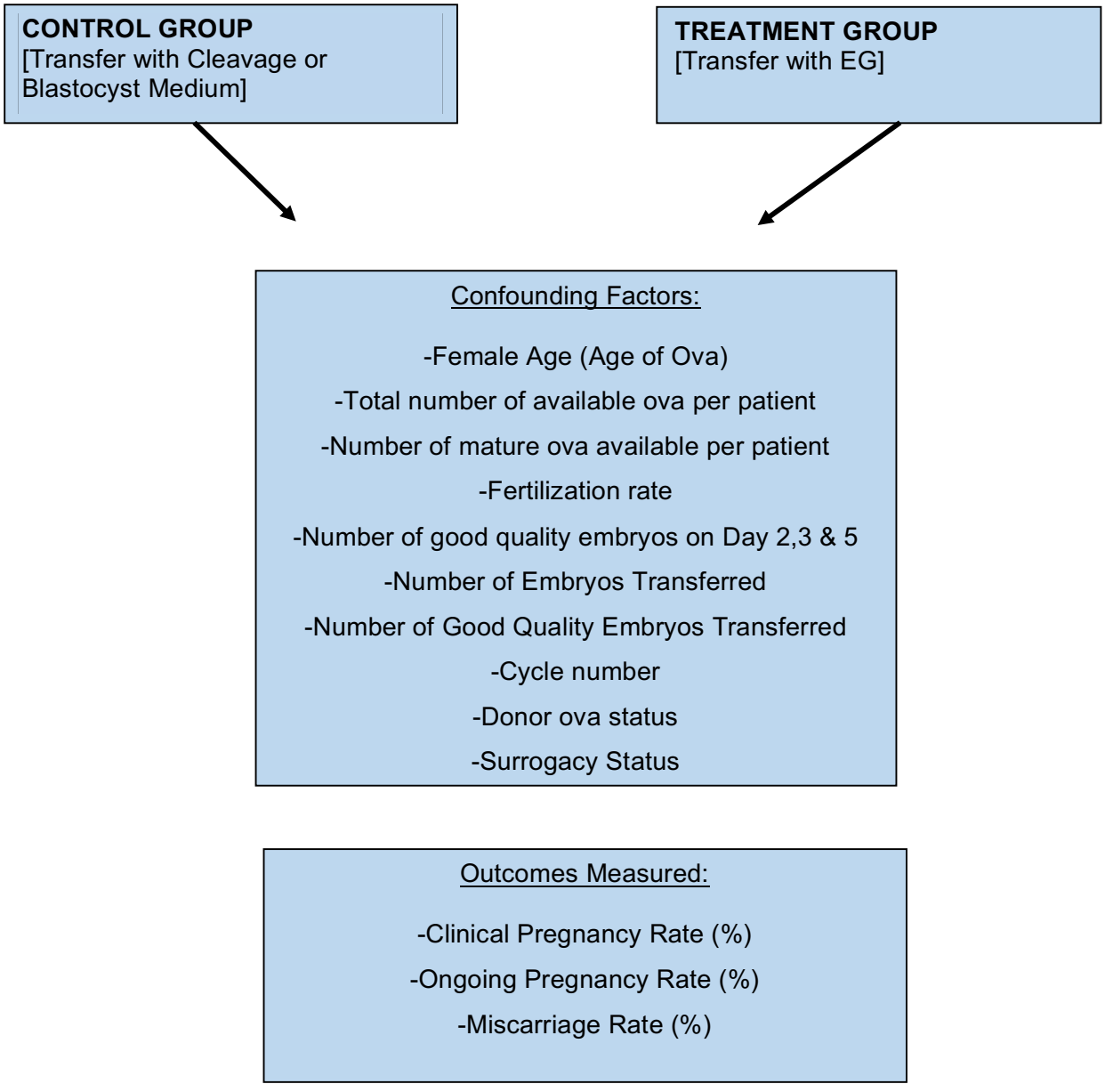
Relevant data was captured and stored to allow statistical analysis of the following confounders between the groups: female age (age of ova), total number of available ova per patient, number of mature ova available per patient, fertilization rate (%), number of embryos transferred, number of of good quality embryos (GQE) transferred and cycle number.

The outcomes measured and statistically analysed to ascertain possible significant differences between the two groups were clinical pregnancy rate, ongoing clinical pregnancy (%) and miscarriage rate (%).

3.1.2 Study Design

Main outcomes to analyse:

To ensure that the control and treatment group outcomes can be attributed to the use of EG alone, the groups were compared in terms of the following confounding factors:



3.1.3 Study Objecftive

Primary objective

To compare the CPR and OPR between the EG transfer patient group and standard transfer patient group.

Secondary objective

To compare the fertilization rate, embryo quality rate [day 2, 3 and 5] and miscarriage rate between EG transfer patient group and standard transfer patient group

- patient demographics (female age, number of oocytes aspirated, embryo quality and number of embryois transferred were included in the analysis,

3.1.4 Definitions of Pregnancy & Miscarriage

Clinical pregnancy (CP) is defined as a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy. It includes ectopic pregnancy and multiple gestational sacs are counted as one clinical pregnancy (Zegers-Hochschild *et al*, 2009). Clinical pregnancy rate [CPR] is expressed per embryo transfer [%]

Ongoing pregnancy (OP) is defined as the presence of fetal heart with or without final outcome after 20 weeks gestation for the purposes of the study. Ongoing pregnancy rate [OPR] is expressed per embryo transfer [%].

Miscarriage was definied as the spontaneous loss of a clinical pregnancy that occurs before 20 completed weeks of gestational age (18 weeks post fertilization) (Zegers-Hochschild *et al*, 2009). Miscarriage rate was expressed per transfer [%].

3.1.5 Data management & Statistical Analysis

All relevant data from eligible cycles was entered on a spread sheet similar to that of the standard clinic database spread sheet.

Data captured included:

1. Clinical pregnancy rate
2. Ongoing pregnancy rate
3. Miscarriage rate
4. Ova age (not female recipient age)
5. Total number of ova retrieved
6. Number of mature (metaphase II) ova retrieved
7. Fertilization rate – ova fertilized/ MII ova retrieved [%]
8. Number of good quality embryos (GQE) on day 2, 3 and 5 respectively
9. Total number of embryos transferred
10. Number of good quality embryos (GQE) transferred
11. Cycle number of treatment

There are some differences in the baseline profiles of the control and treatment groups; this can be explained by the nature of the study; a comparative study using retrospective data as control and prospective data as treatment group. The difference in clinical profile between the two groups was taken into account when assessing potential confounding factors.

A logistic regression model of clinical pregnancy on group adjusted for other factors and confounders was used to estimate the adjusted odds ratio and 95% confidence intervals for EG. Possible interaction of factors with EG use was also investigated. The additional factors included in the model were; the number of good quality embryos returned (categorical); day of embryo transfer, donor ova status, age of ova, number of good quality embryos transferred and total number of embryos transferred. The number of ova available per patient was not adjusted for because that information was not captured for those patients who underwent frozen embryo transfers (17% of patient data). The missing data was accounted for and was determined not to

have any serious bias or effect, the model without adjustment for number of ova was not significantly different from model without this covariate. This regression model approach is a powerful tool to compensate for the observational nature of the study by trying to imitate the conditions and advantages associated with a randomized controlled trial.

The statistical tests used for the purpose of the study included the following;

- Analysis of variance (ANOVA):
  ANOVA is a collection of statistical models used to analyse and quantify intergroup and intragroup variability

- Pearson Chi^2 test
  A chi-square test for independence or association used to discover if there is a relationship between two categorical variables.

- A quantile non-parametric logistic regression model
  Quantile regression is a type of regression analysis used to obtain a comprehensive analysis of the relationship between variables. It is a powerful tool to adjust for confounding and can be used to closely simulate the conditions of randomization.

- Fisher's exact test
  -Fisher's exact is a statistical significance test to determine if there are non-random associations between two categorical variables; it is more accurate than chi-squared test and is used when sample sizes are too small for chi-squared analysis.

3.1.6 Procedures

All ART procedures were similar for the retrospective and prospective study except for embryo transfer methodology in the prospective study.

Ovarian stimulation

Female partners underwent controlled ovarian hyper-stimulation according to standardized stimulation protocols. The appropriate stimulation protocol is determined based on patient presentation and medical history, namely; diagnosis, physiology, endocrinology and age. Standard stimulation protocols include; i. Short Protocol, ii. Long Protocol, iii. Natural Cycle, iv. Modified Natural Cycle and v. Antagonist Protocol. Stimulation protocol sub-types were designated individualized codes for the purpose of accurate data collection.

Stimulation involves administration of gonadotrophin-releasing hormone agonist (GnRHa) followed by human menopausal gonadotrophins (HMG) and/or pure follicle stimulating hormone (FSH) from cycle day 3. Follow-up procedures included estradiol determinations and ultrasonographical measurements of the Graafian follicle. Once the leading follicle had reached a diameter of 18mm. Human chorionic gonadotropin (HCG) was administered to induce ovulation.

Aspiration and gamete handling

Standard procedures were used [Appendix III & XII].

Semen preparation

Sample characteristics such as sperm count and motility determined the semen preparation method used. Standard wash and swim-up techniques, using HEPES buffered sperm preparation medium, were used to isolate motile spermatozoa during semen preparation when possible. Gradient (90%, 45%) centrifugation method was used for samples with parameters below reference ranges as determined by the World Health Organisation (WHO, 2010). [Appendix XI].

ICSI Method

Refer to Appendix I & II.

PICSI® Method

Refer to Appendix VII.

IMSI Method

Refer to Appendix VIII.

Embryo culture and evaluation

Standard protocol descriptions for Embryo Culture & evaluation can be observed in Appendix III.

Embryo transfer

Standard protocol was used but with modifications for study protocol.

EG Treatment Group: Embryo transfer

Embryos selected for transfer were placed in 0.5 ml CO_2 equilibrated, warmed [37°C] EG medium for at least 10 minutes, but not longer than 30 minutes, before embryo transfer. Thereafter standard routine embryo transfer was followed. [Appendix XIV]

Control Group: Embryo transfer

Embryos selected for transfer were placed in 1ml CO_2 equilibrated, warmed [37°C] culture medium (Cleavage or Blastocyst medium, SAGE) before embryo transfer. Thereafter standard routine embryo transfer protocol was followed. [Appendix XIV]

Embryo Quality

Embryo quality was evaluated according to standard protocol in the clinic based on the work of Veeck, 1999 & Veeck *et al,* 2003. [Appendix IV]. Blastocyst grading is according to a modified Gardner & Schoolcraft grading system [Appendix V].

Embryo Vitrification & Warming

Refer to Appendix XIII.

Pregnancy

Clinical pregnancy (CP) is defined as a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy. It includes ectopic pregnancy and multiple gestational sacs are counted as one clinical pregnancy (Zegers-Hochschild *et al*, 2009).

Ongoing pregnancy (OP) is defined as the presence of fetal heart with or without final outcome after 20 weeks gestation for the purposes of the study.

Miscarriage

Miscarriage was definied as the spontaneous loss of a clinical pregnancy that occurs before 20 completed weeks of gestational age (18 weeks post fertilization) (Zegers-Hochschild *et al*, 2009). Miscarriage rate was expressed per transfe [%].

Consent Forms

Patient information leaflet and consent forms were approved by the Health Research Ethics Committee [Appendix IX]. (Ethics Reference #: S15/04/090).

3.2 Results

3.2.1 Patient Population

The patient population included in the prospective study was comprised of 870 patients. The respective sample sizes of relevant treatment groups were; 197 patients in the EG treatment (EG embryo transfer) group and 673 patients in the control (classical embryo transfer) group. The exclusion criteria were designed to allow for a comparison of embryo transfer with and without high concentrations of HA. The comparison of embryo transfers with or without EG as transfer medium was the primary focus of this portion of the study. The two groups were not matched, but logistic regression analysis was performed to adjust for all possible confounding influences.

3.2.2 Subgroup Analysis: Estimating possible differences between groups

The resulting differences between the control and EG subgroups regarding oocyte age, number of embryos transferred donor ova status and cycle number is presented in Table 3.1.

When comparing the number of patients younger than 36 and those 36 and over, the age distribution was comparable and not significantly different, between the control and EG groups; 61.72% of control and 61.72% of EG patients were younger than 36 years, (p=0.397). There was a significant difference in control and EG group profiles in terms of number of embryos transferred per patient (p=0.0027). In the control group, 6.82% more transfers with 2 or more embryos were performed. Also, in the control group, only 17.80% of the patients made use of donor ova while 26.90% of the EG group patients used donor ova. This 9.1% difference in the number of patients who made use of donor ova in respective groups was significant (p=0.005). Cycle number was not available for all patients included in the study, however no significant difference in profile of cycle number between groups was detected with the available data, which included 686/870 of the total patients; 42.35% of the control patients had two or more cycles compared to 46.67% in the EG group (p=0.346).

Table 3.1 Subgroup analysis comparing control and EG clinical profiles

Variable	Group	Control	EG	Total
Age of Ova	<36 years	61.72% [416/674]	58.38% [115/197]	60.96% [531/871]
	≥ 36 years	38.28% [258/674]	41.62% [82/197]	39.04% [340/871]
Number Transferred	1 embryo	16.02% [108/674]	22.84% [45/197]	17.57% [153/871]
	≥ 2 embryos	83.98% [556/674]	77.16% [152/197]	82.43% [718/871]
Donor Ova Status	No donor ova	82.20% [554/674]	73.10% [114/197]	80.14% [698/871]
	Donor ova	17.80% [120/674]	26.90% [53/197]	19.86% [173/871]
Number of ART Cycles Attempted	First cycle	57.65% [309/536]	53.33% [80/150]	56.71% [389/686]
	≥ 2 cycles	42.35% [227/536]	46.67% [70/150]	43.29% [297/686]

3.2.3 Measured Outcomes

Clinical Pregnancy [CP] (Fetal Sac) outcome: Unadjusted, Crude Comparison

A Pearson Chi^2 Test was used to analyse and compare the CP outcomes of control and EG treatment groups. The analysis was initially performed on the crude, unadjusted data. CP data was available for 100% of the patients [673/673] in the retrospective control group and in 96.1% [197/205] for the EG group. There was a statistically significant difference (p=0.004) in the number of fetal sacs among patients in the control group compared with that of patients in the EG group (Table 3.2). Interestingly, the proportional twin-rate in the EG group (13.20%) was also more than double that of the control group (6.39%).

Table 3.2 Number of fetal sacs in the control and EG groups.

Fetal Sac (#)	Control	EG	Total
0	67.61% [455/673]	58.88% [116/197]	65.63% [571/870]
1	25.56% [172/673]	26.40% [52/197]	25.75% [224/870]
2	6.39% [43/673]	13.20% [26/197]	7.93% [69/870]
3	0.45% [3/673]	1.52% [3/197]	0.695 [6/870]

A binary analysis of CP, regardless of fetal sac number followed (Table 3.3). The CP rate differed 8.73% between the EG (41.12% [81/197]) and the control groups (32.39% [218/673]). Interestingly, the proportional twin-rate in the EG group (13.20%) was also more than double that of the control group (6.39%). The crude odds ratio was 1.46 (95% CI: 1.05 to 2.02) indicating a 46% greater likelihood in achieving CP following embryo transfer with EG medium. This unadjusted comparison of raw data showed a significant difference (p=0.023) in the CP profiles of the two groups.

Table 3.3 CPR, regardless of fetal sac number, in control and EG groups.

Fetal Sac	Control	EG	Total
None	67.61% [455/673]	58.88% [116/197]	65.63% [571/870]
Any number	32.39% [218/673]	41.12% [81/197]	34.37% [229/870]

Clinical Pregnancy [CP] (Fetal Sac) outcome: Adjusted for covariates

The subgroup analysis is the underpinning of the final model, in none of the factors that are part of the model are there any subgroup effects thus, only the main effects model is presented; where the effect of EG is adjusted for covariates: day of ET, age of ova, number of GQE ET, total number ET and donor ova status.

The results of the adjusted logistic regression model are presented in Table 3.4. The adjusted OR for EG is 1.37 (95% CI: 0.97 to 1.96) indicating a 37% greater likelihood in resultant clinical pregnancy than the control but the adjusted effect is no longer significant (p=0.078). The age of ova (p=0.785), and the total number of embryos transferred (p=0.238) had no significant effect on CP either. The day of embryo transfer (p=0.022), donor ova status (p=0.040) and number of good quality embryos transferred (p=<0.001) were all significant factors in the determination of CP outcome. The use of donor ova was associated with a 66% greater likelihood in achieving CP. The number of GQE returned was specifically strongly associated with higher odds for a positive CP, increasing in likelihood as number transferred increased. A locally weighted scatter plot smoother (LOWESS) represents this relationship (Figure 3.1).

Table 3.4 OR outcomes for CP in the adjusted, logistic regression model.

Variable	Odds Ratio [OR]	95% Confidence Interval	p-value
EG (ref Control)	1.37	0.97 to 1.95	0.078
Day of Embryo Transfer	1.17	1.02 to 1.33	0.022
Donor Ova	1.66	1.02 to 2.70	0.040
Age of Ova	0.99	0.96 to 1.03	0.785
Number GQ ET (ref 0)			<0.001
1 GQ ET	2.73	1.48 to 5.02	0.001
2 or more GQ ET	7.14	4.02 to 12.70	<0.001
Total Number ET	0.86	0.67 to 1.10	0.238

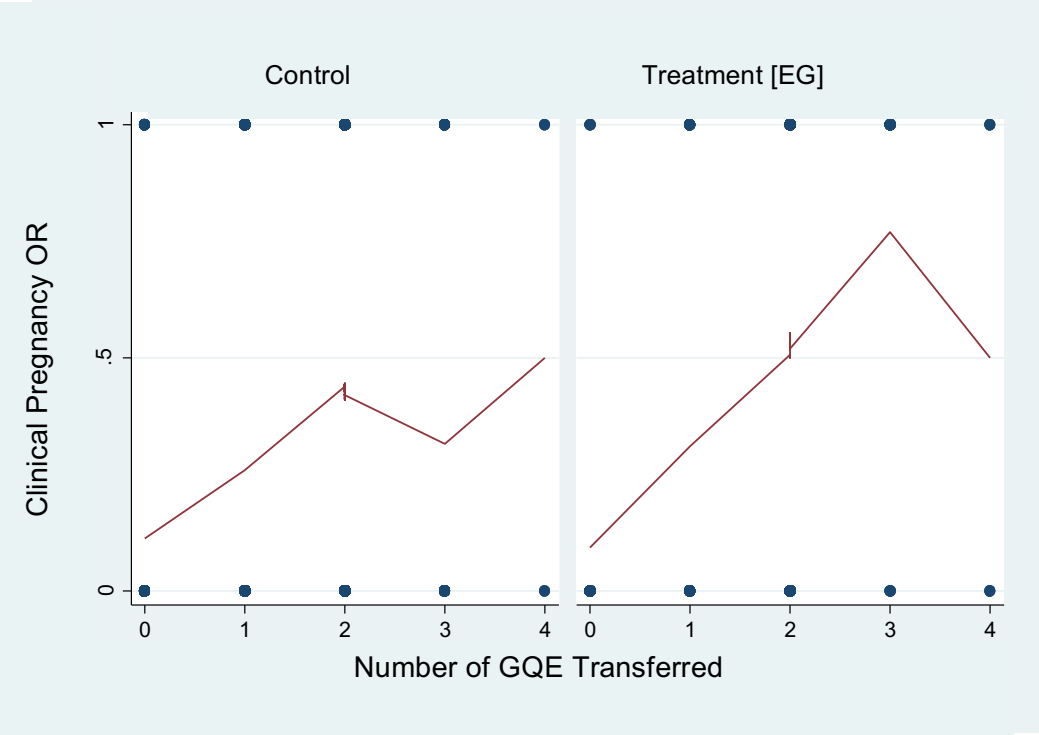


Figure 3.1 A LOWESS (Locally Weighted Scatterplot Smoothing) graph representing OR for CP according to the number of GQE transferred in the control and EG groups.

The absence of an interaction between the number of good quality embryos returned and the use of EG or not is reflected in Table 3.5. Although the observed profile of CP differs there is no statistical difference (p=0.4946), hence the effect of EG was estimated without an interaction term in the model.

Table 3.5 CPR according to number of GQE transferred in the control and EG groups.

	Control	**EG**
Number Good Quality Returned	**Fetal Sac(s)**	
0	9.38% [3/32]	11.32% [12/106]
1	31.03% [18/58]	25.91% [57/220]
2 or more	56.07% [60/107]	43.06% [149/346]
Total	41.12% [81/197]	32.44% [218/672]

Ongoing Pregnancy Rate [OPR] (Fetal Heart): Unadjusted, Crude Comparison

A Pearson Chi^2 Test was used to compare the crude unadjusted data for OPR (fetal hearts) resulting in control and EG groups. Results were similar to that of CPR (fetal sacs). However, the number of fetal hearts detected per patient within control and EG groups was not significantly different (p=0.059) (Table 3.6) although the proportional twin rate in the EG group (13.71%) was once again almost double that of the control group (7.88%).

Table 3.6 Number of fetal hearts detected per patient in control and EG groups.

Fetal Heart	Control	EG	Total
0	68.80% [463/673]	60.91% [120/197]	67.01% [583/870]
1	23.03% [155/673]	24.87% [49/197]	23.45% [204/870]
2	7.88% [53/673]	13.71% [27/197]	9.20% [80/870]
3	0.30% [3/673]	0.51% [1/197]	0.34% [3/870]

The binary analysis of OPR (unadjusted) regardless of fetal heart number is presented in Table 3.7. The CP rate differed 7.893% between the EG (39.09% [77/197) and the control (31.20% [210/673]) groups. The crude OR was 1.41 (95%CI: 1.02-1.97) and was statistically significant (p=0.039) indicating a 41% greater likelihood in achieving an OPR when EG was used as embryo transfer medium. The analysis indicated that the differences in OPR was statistically significant between two groups (p=0.038).

Table 3.7 OPR, regardless of fetal heart number in control and EG groups.

Fetal Heart	Control	EG	Total
No Fetal Heart	68.80% [463/673]	60.91% [120/197]	67.01% [583/870]
Any number of Fetal Hearts	31.20% [210/673]	39.09% [77/197]	32.99% [287/870]

Ongoing Pregnancy Rate [OPR] (Fetal Heart): Adjusted for covariates

The effect of EG was adjusted for covariates: day of ET, ova age, number of GQE ET, one GQE ET, ≥ 2 GQE ET, total number ET and donor ova.

A logistic regression model results for OPR are shown in Table 3.8. The adjusted OR for EG is 1.34 [95% CI: 0.94 to 1.91], indicating a 34% greater likelihood in achieving OPR compared to the control group. The adjusted effect is not significant (p=0.108). The age of ova was not significant (p=0.513) nor was the total number of embryos transferred (p=0.114) or donor ova status (p=0.087). The day of embryo transfer was a significant determinant for OPR outcome (p=0.027). The number of GQE returned is strongly associated with OPR, with a much higher OR [OR=6.64 (95% CI: 3.73 to 11.82)] for OPR when 2 or more GQE are returned (p=0.001) compared to none.

Table 3.8 OR outcomes for OP in the adjusted, logistic regression model.

Variable	Odds Ratio	95% Confidence Interval	p-value
EG (ref Control)	1.34	0.94 to 1.91	0.108
Day of Embryo Transfer	1.16	1.02 to 1.32	0.027
Donor Ova	1.53	0.94 to 2.50	0.087
Age of Ova	0.99	0.95 to 1.02	0.513
Number GQ ET (ref 0)			<0.001
1 GQ ET	2.52	1.37 to 4.66	0.003
2 or more GQ ET	6.64	3.73 to 11.82	<0.001
Total Number ET	0.82	0.63 to 1.05	0.114

Miscarriage rate: Unadjusted Comparison

A Pearson Chi^2 Test was used to compare the crude unadjusted data for miscarriages in the control and EG groups. The study used a unified definition of pregnancy loss for all types of miscarriages, at various stages of pregnancy, to form a single binary outcome. The miscarriage rates in the control (5.49%) and the EG groups (6.60%) was not significantly different (p=0.556). The crude OR [OR= 1.22 (95% CI: 0.63 to 2.34)] for miscarriage showed no significant difference in likelihood of miscarriage between groups (p=0.557), thus no further adjustment was made (Table 3.9).

Table 3.9 Miscarriage rate, regardless of pregnancy stage, in control and EG groups.

Miscarriage	Control	EG	Total
No spontaneous abortion	94.51% [637/674]	93.40% [184/197]	94.26% [821/871]
Miscarriage	5.49% [37/674]	6.60% [13/197]	5.74% [50/871]

3.3 Discussion

The study found significance in favour of EG only in crude analysis of the data and when no adjustments were made for confounding. Unadjusted comparison in respect of CPR (fetal sac rate) and OCPR (fetal heart rate) both indicated significant differences among control and EG group- outcomes. After adjustment for confounding factors - oocyte age, day of transfer, total number of embryos transferred, number of GQE transferred and donor ova status however, regression analysis showed the differences in CPR and OCPR were no longer significant. In the analysis of CPR, the day of embryo transfer, use of donor oocytes and number of GQE were significant confounding factors. Ova age and total number of embryos transferred were not significant factors. For OCPR the day of embryo transfer and number of GQE stayed significant confounding factors, but ova age, total number of embryos transferred and use of donor oocytes were not significant.

Subgroup analysis

A subgroup analysis was performed for oocyte age, number of embryos transferred, donor ova status and cycle number to determine if the two groups were comparable. The age distribution profiles of the two groups did not differ significantly, nor the profiles of cycle number. There was a significant difference in group-profile's in terms of number of embryos transferred per patient. In the control group, 6.82% more transfers with two or more embryos were performed. In the treatment group, 26.90% of the patients made use of donor ova compared to 17.80% of the control group. This 9.1% difference in the number of patients who made use of donor ova in respective groups was also significant. In the final regression analysis, the number of embryos transferred and the donor status were included as confounding factors and adjusted for.

Clinical Pregnancy Rate (CPR) outcomes and confounding factors

The unadjusted, crude comparison indicated a significant improvement in CPR when EG was used as transfer medium (41.12% vs. 32.39%). The crude odds ratio indicated a 46% greater likelihood in achieving CP following embryo transfer with EG. When a logistic regression model with odds ratios was applied to adjust for potential confounders, differences in group outcomes were no longer significant in terms of CPR. The adjusted odds ratio indicated 37% greater likelihood in achieving CP following embryo transfer with EG but the adjusted effect was no longer significant. The day of embryo transfer had a significant effect on the likelihood of achieving CP – the odds ratio indicated a 17% greater likelihood in achieving CP with Day 5 embryo transfers. The use of donor ova and the number of good quality embryos transferred

were also associated with significantly higher odds of achieving CP. The use of donor ova was associated with a 66% greater likelihood in achieving CP while the likelihood of CP increased directly with the number of GQE returned. When one GQ embryo is transferred, it results in a 273% greater likelihood in CP compared with the transfer of a poor quality embryo. In contrast, the effects of ova age and total number of embryos transferred were not significant.

Ongoing Pregnancy Rate (OPR) outcomes and confounding factors

The unadjusted comparison indicated a significant improvement in OPR when EG was used as transfer medium (39.09% vs. 31.20%). The crude odds ratio indicated a 41% greater likelihood in achieving OPR following embryo transfer with EG. When adjustments were made for confounding using the same logistic regression model with OR, OPR differences were also no longer significantly affected by the use of EG. The adjusted OD indicated a 34% greater likelihood in achieving OPR in the EG group compared to control group. The day of embryo transfer was found to have a significant interaction with the effect of EG, where day 5 transfer was associated with a 16% greater likelihood in OPR. Again, the number good quality embryos transferred was strongly associated with ongoing pregnancy, with much higher odds for ongoing pregnancy when two or more GQE are returned. However, the effect of ova age was not significant, nor was the total number of embryos transferred or donor ova status.

Miscarriage Rate

The unadjusted comparison in miscarriage rate in the C and EG groups (6.60% vs. 5.49%) indicated no significant difference in group profiles, thus no adjustments were made. A unified definition of pregnancy loss for all types of miscarriages, at various stages of pregnancy, was used to form a single binary outcome.

Confounding Factors

The confounding factors oocyte age, total number of embryos transferred and number of GQE transferred have been discussed in the retrospective analysis section. Younger age of oocytes are associated with better prognosis as oocyte abnormalities and aneuploidy become increasingly common with increasing age. (Armstrong, 2001, Kuliev *et al*, 2003). The transfer of two or more embryos results in higher pregnancy rates than single embryo transfers (Martikainene *et al*, 2001). The transfer of GQE is positively associated with pregnancy outcome, likelihood of positive pregnancy outcome increases directly as number of GQE increases (Rienzi *et al,* 2005). In the present study, the number of GQE transferred was a

significant factor in determining the likelihood of positive CP and OP and this outcome agrees with the literature. In contrast with available literature, the present study found no significance associated with age of ova nor with total number of embryos transferred. This may be explained by the high number of patients that made use of donor ova, associated with improved outcome (Sauer *et al,* 1990 & 1992). The reason total number of embryos transferred was not a significant factor may be the result of confounders not adjusted for in the study; potentially the etiology of infertility.

Study outcomes confirmed evidence in literature that the day of transfer can have an effect on outcomes. Studies have illustrated a favourable association between blastocyst culture to day 5 & 6 and live birth rate, particularly for patients with good prognosis (Blake *et al*, 2004). The present study indicated greater likelihood in achieving OP with day 5 transfers similar to what the literature indicates. Blastocyst, day 5, transfer increases the live birth rate per couple (Blake *et al.,* 2007) and ongoing and cumulative pregnancy rates in comparison with day 3 transfers where at least 4 embryos were available (Fernandez-Shaw *et al*, 2015). The influence of **donor ova use** on outcome has been investigated extensively. An American retrospective record review of 27 959 fresh donor oocyte IVF cycles found that with the use of donor ova women between age 39 and 45 were able to achieve similar implantation, CP and live birth rates compared with their younger counterparts (Yeh *et al*, 2014). The present study found a significant benefit in donor ova use in terms of CP with a 66% increase in the odds of achieving CP but found no significant difference in likelihood of OP, possibly due to the fact that EG isn't specifically indicated for cycles making use of donor ova but rather for a specific sub-group of patients with particular etiology of infertility.

Comparison of Results with the Relevant Literature

The results of the current study showed no *significan*t benefit for EG over classical transfer medium when confounding factors were considered in logistic regression analysis. EG did however result in an improved CPR and OPR in crude analysis (41.12% vs. 32.39% and 39.09% vs. 31.20% respectively). The use of EG remains controversial among ART professionals, as it is difficult to conclude the favourable role of EG as routine transfer medium in all treatment cycles.

A prospective, randomized, controlled study analysed the use of HA-enriched transfer medium in a total of 825 day 3 and 457 day 5 embryo transfer cycles. Pregnancy rates were compared in terms of day of embryo transfer, women's age, quality of embryos transferred and presence of previous implantation failures to adjust for confounding. The study found significant increases in CPR and implantation rate with the use of HA-enriched transfer medium. The

beneficial effect was greater in patients over 35 years of age, women who had previously failed cycles and women with poor embryo quality (Urman *et al*, 2008). A Cochrane review of adherence compounds in embryo transfer media for ART included 15 studies evaluating HA. Although there was no evidence of a treatment effect on live birth rate, there was evidence of significant treatment effect on both CPR and multiple pregnancy rate. (Bontekoe *et al*, 2010). A 2014 meta-analysis on almost 10 000 embryo transfers carried out at various Japanese clinics further confirmed the findings of the 2010 Cochrane report evaluating the use of high concentrations of HA in embryo transfer medium. The meta-analysis concluded that the use of EG as transfer medium resulted in a significant increase in both implantation and pregnancy rates. Miscarriage rates were not significantly different (Hashimoto *et al*, 2014). In 2014, the Cochrane Collaboration published an update of their first meta-analysis also entitled "Adherence compounds in embryo transfer media for assisted reproductive technologies (Review)". The updated review included 16 truly randomized controlled trials with a total of 3898 total participants. Out of those included, 14 studies reported CPR as endpoint and 6 studies also included LBR. The use of EG for embryo transfer, when compared with the use of culture medium containing little or no hyaluronic acid, resulted not only in an improvement of successful implantation rates but also in an overall increase of 8% live birth rates, regardless of embryo transfer day (Bontekoe *et al.,* 2014). The results from the crude unadjusted analysis in the present study agreed with these findings, but once adjustments were made for confounders, improvements in CP and OP rates were no longer significant. Individual studies identify and adjust for different confounders, thus the results are causally linked with the exclusion criteria and confounder adjustments made. Additionally, patients were accepted into the EG treatment group regardless of indication. EG is not indicated for a general patient population. The potential beneficial effect of EG is applicable for patients of advanced maternal age (poor embryo quality), previously failed cycles and/or recurrent implantation failure.

In 2005, a study designed to evaluate the usefulness of EG in improving pregnancy rates included 310 fresh embryo transfers, 154 of which were transferred following incubation in the HA-enriched medium. Possible confounding factors such as age, duration of infertility, previous IVF cycles, total gonadotropin dose, oocyte number and number of embryos transferred were similar in the control and treatment groups. The resulting pregnancy rates were not significantly different and they concluded the use of EG in fresh transfers did not improve resulting pregnancy rate (Ravhon *et al*, 2005). Additional abstracts from oral and poster presentations at the American Society for Reproductive Medicine (ASRM) scientific congress & expo were published by Fertility and Sterility in September 2013. A study from the Center for Reproductive Medicine of New Mexico, Albuquerque including 179 frozen embryo replacement cycles, reported that the use of EG as post-thaw culture and transfer medium did not improve the

measured clinical outcomes in frozen embryo replacement cycles. The measured outcomes included clinical and ongoing pregnancy, and implantation rate per embryo transferred (Said *et al,* 2013). A prospective case-control study conducted at the assisted reproductive centre of a tertiary care hospital involved 84 women undergoing fresh non-donor IVF or ICSI cycles. In the control group (*n*=42), embryos were transferred to conventional blastocyst culture medium and those in the treatment group (*n*=42) were transferred into 50µL of EG for 10 min prior to transfer inside the uterine cavity. In an effort to limit possible confounding factors the exclusion criteria included women age >35 years, those with poor ovarian reserve, and possible causes of failure of implantation such as diabetes mellitus, hypertension and autoimmune diseases. The resulting CPR in the treatment group was 7% higher than that of the control group; the difference was not statistically significant. A significant difference in CPR was however observed in a subset (n=12) of treatment patients who had previous failed ART cycles. In the treatment group, 50% [6/12] of patients achieved successful implantation where none of the patients [0/11] in the control group did (Singh *et al*, 2015). A 2015 cohort study performed on 229 retrospectively enrolled patients also found no improvement in ART outcomes following the routine use of EG in all treatment cycles (Safari *et al*, 2015).

Studies that have demonstrated marginal improvements in achieving clinical pregnancy and reducing miscarriage rate with the use of EG were likely subject to a number of confounders, not all studies were randomized controlled and regression analysis was not applied in all cases to account for the limitations of study design. The present study also concluded no significant improvement in measured outcomes when EG was used for all patient cycles regardless of infertility etiology and when confounding factors were adjusted for.

Limitations of the study

The results differ from the hypothesis in that a statistically significant improvement in CP and OP rates, after adjusting for confounding factors, did not result from the use of EG embryo transfer medium. Miscarriage rate was also not significantly altered by the application of EG. The results of the present study may be due to the following limitations.

The lack of randomization or pairing, although a logistical regression model with odds ratios was used to compensate for the observational nature of the study by trying to mimic the conditions and advantages associated with a randomized controlled trial. The small sample size limits the robustness of the study; the sample size was affected by the necessary exclusion criteria to limit confounding as well as the number of patients initiating cycles during the study period. There were also challenges in obtaining informed consent from all eligible

patients. Intern medical scientists are limited in their interaction with patients at the private practice and therefore had to rely on the help of their colleagues. A number of eligible patients also could not be included as their transfers took place before embryos had been in EG for the stipulated 10-minute minimum exposure. The success of a study such as this, in a private facility, not only depends on the commitment of the investigator but also on the participation of other members of the practice. Eligible patients in the treatment group who did not receive EG due to initial stocking difficulties and lack of availability also had to be excluded.

Due to insufficient record keeping, the inability to identify patients for whom EG is indicated, such as those with recurrent implantation failure or repeated miscarriage, made it impossible to study this subgroup. The application of EG in all cycles, despite not being indicated for the general infertile population, was however done. The system in place for data capturing and medical record keeping also makes the etiology of infertility difficult to determine. Patients of advanced age who make use of donor ova have their diagnosis recorded as “Donor Ova” rather than advanced maternal age for example. These inconsistencies meant that etiology of infertility, a potential confounder, could not always be adjusted for.

Conclusion & Recommendations

The results from the present study demonstrate that the routine application of EG as embryo transfer medium in all ART cycles does improve CPR and OPR but its significance disappears when known pregnancy confounding factors are adjusted for. However, literature suggests its usefulness in the treatment of couples presenting with specific indications of infertility such as those who have had recurrent implantation failure, repeated miscarriages and some studies also indicate its usefulness in patients with tubal factors (Valojerdi *et al*, 2006). Clinical significance and statistical significance may differ in a unique clinical setting. A 37% greater likelihood for CP and and 41% greater likelihood for OP (although not statistically significant) for adjusted results may still be considered clinically significant if the use of a particular technique is also the safest and most cost effective option available. The hypothesis is one of superiority & difference. One could also consider it from a perspective of equivalence. Given the confidence intervals and assuming equivalence limits of 10% the estimated confidence intervals between EG and the standard transfer medium show equivalence since the limits lie within -10 and +10%.

The improvement in implantation rates observed in patients with previous failed cycles and recurrent implantation failure in literature, demands further investigation. The present study, as well as available literature, confirms a general increase in the likelihood of multiple pregnancy

resulting following EG [see Table 3.2 and 3.6] and it is therefore advisable to reduce the number of selected embryos for intrauterine transfer (or implement a policy of single embryo transfers) when using this transfer medium. A follow up study might include, (i) only patients with a specific diagnosis (advanced age, previously failed cycles, poor quality embryos as indicated by literature), (ii) randomization, (iii) analysis of implantation rates and live birth rates and (iv) a commitment to culturing embryos for transfer in EG for the prescribed 10 to 30 minutes period prior to initiating transfer. A general awareness and commitment to ongoing research projects is necessary in order to effectively coordinate clinic staff and ensure the complete and accurate collection of data required for optimized study conditions.

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Appendices

APPENDIX I Drs Aevitas Fertility Unit Standard Protocol: ICSI

All forms and documents are prepared.
The thorough check of the patient's file and record is completed to eliminate all possible uncertainties/queries.

Semen preparation

See section on semen preparation methods

Aspiration

Medium preparation – previous day

See section on medium preparation

Ovum Pick up

- Check suction pump (100-120 mmHg)
- Prepare glass polished pipettes for pick up
- Place pick-up tubes in heated block
- Hand theatre medium tube to sister when needed
- Place aspirated follicular fluids in heated block and examine for oocyte-cumulus complexes using a large Petri dish on a heated stage (37-40ºC) of a dissection microscope
    - Note obvious abnormal features and maturities
- Put the complexes (with as little as possible blood and medium) in the pick-up tube
- When done, rinse all complexes in small Petri dish with gassed fertilization medium – check number obtained
- Leave in fertilization medium in CO_2 incubator until time for denuding of oocytes
    - Try to do denuding ±38 hours post HCG injection

Denuding of oocytes

- Prepare pipettes for the process: fire polished glass Pasteur pipettes, hand drawn glass pipettes and the Cook stripper (Marcus Medical) pipette
- Prepare a 4 well NUNC dish for denuding: **(USE ONE DISH FOR EVERY 8 OOCYTES)**

- In well 2, 3 and 4 place ± 0.8 – 0.9 ml warmed HEPES buffered flushing medium (Quinns, Cooper Surgical)

- In well 1 place 0.6 ml HEPES buffered flushing medium and add 0.3 ml hyaluranidase [80 UI/ml] (Quinns, Cooper Surgical)

- Place in the incubator [without CO_2] at 37ºC for ± 10 minutes to reach 37ºC

- Place a predetermined number of oocyte/cumulus complexes in well 1, wait for ± 30 seconds

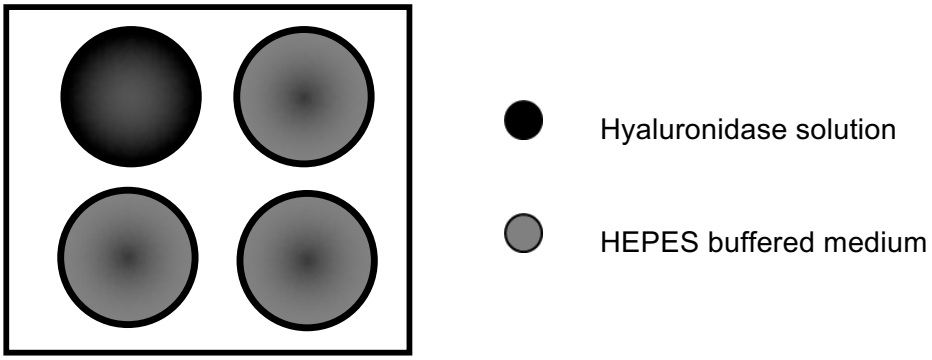
- Gently flush the complexes with a standard fire polished pipette until all cumulus cells are digested (oocytes with corona cells and small number of cumulus cells form “fluffy balls”)

    - If complexes stay intact use two hypodermic needles to “tease” oocytes from the complexes

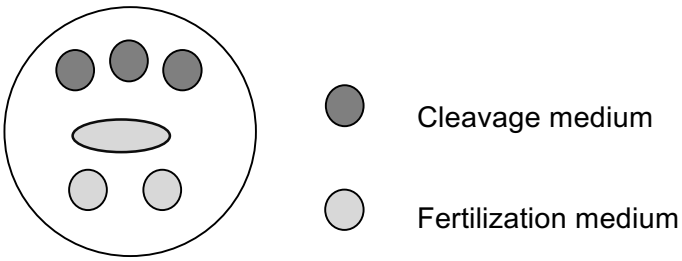
- Use the same fire polished pipette and transfer the oocytes with as little as possible hyaluronidase solution to well 2

- Now flush oocytes individually with a big [170-200um] pulled glass pipette to remove some of the loose cumulus cells and transfer to well 3

- In well 3 start the stripping using the plastic Cook denuding pipette [130um] (Marcus Medical) – try to remove all corona cells to allow for evaluation of oocyte maturity
    - Make sure that the pipette works correctly before oocytes are aspirated
    - If oocytes seem stuck in pipette – blow out into one of the wells using a syringe and adapter



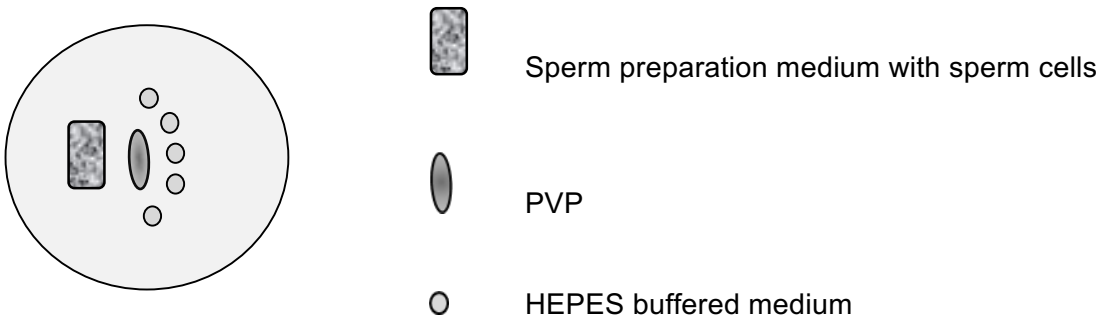
- Place the denuded oocytes into a pre-prepared holding or “rugby ball” dish, into the elliptical fertilization wash medium drop.
- [this dish is prepared the previous day and cultured at 6% CO_2/37ºC – drops are covered with oil – Quinns- Cooper Surgical)



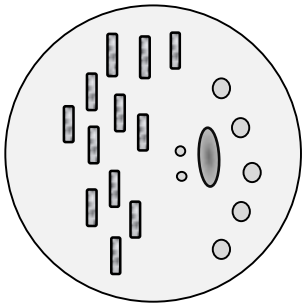
- Now determine the maturity of the oocytes and transfer to the clean fertilization drops – all MII oocytes to the left drop and all MI and GV to the right drop
- Culture until injection
- Complete all forms
- Set up the inverted microscope for ICSI [heated stage, holding and injection pipettes, manipulators]

Injection

- For ICSI with **ejaculated semen**, prepared the following injection dish:



- For ICSI with **testicular biopsy sample or severe oligiozoospermia semen**, prepare the following injection dish:



- Incubate for ±30 minutes at 37ºC [no CO_2]

Sperm Immobilization

- Add sperm cells to the sperm preparation medium
- Select motile, morphological normal spermatozoa form the drop with the ICSI injection pipette and immobilize the sperm cell
    - Collect enough sperm cells for the injection procedure.

[*A detailed description of the immobilization process is given in Appendix II "ICSI handbook addendum"*]

Sperm injection

- Place MII oocytes to be injected in the oocyte drops (2 per drop)
- Select an immobilized sperm cell and carefully inject oocyte

[***for a detailed description of the injection process, see "ICSI handbook addendum"***]

- Continue until all oocytes are injected
- Transfer back into the elliptical drop (fertilization) to wash and then to the cleavage drops for overnight culture (6% CO_2/37ºC
- Do injection ± 40 hours post HCG administration if at all possible

APPENDIX II Drs Aevitas Fertility Unit: ICSI Handbook

Intracytoplasmic sperm injection

The microinjection procedure is performed on an inverted microscope equipped with:

1. A heated microscope stage (37°C).
2. Two coarse and fine control manipulators.
3. Two micro injectors, one for the holding pipette and the other for the injection pipette.

The holding pipette is inserted into the pipette holder and manually positioned so that the angled section is perpendicular to the microscope stage. The pipette is positioned using the coarse manipulators. The microinjection pipette is inserted into the pipette holder and manually positioned so that the angled section is at angle of approximately 20° to the microscope stage. The angle is to ensure that the tip of the microinjection needle will touch the surface first. It must also be ensured that the two pipettes (holding and injection) move in line with each other.

The microinjection dish is made according to the configuration of your microinjection system and the experience of the technician. The dish must, however, contain certain basic elements; an oocyte droplet, spermatozoa, PVP droplet.

Selection and immoblization of sperm

The microinjection pipette is first carefully lowered into the clean PVP droplet. Before the manipulation of sperm is attempted PVP medium is repeatedly aspirated and expelled to coat the inside of the microinjection pipette. A large enough volume (for optimum control) of PVP is aspirated and the pipette is lifted and moved to the sperm storage droplet.

The motile spermatozoa pipetted into the storage droplet swim to the outer perimeter of the droplet. When selecting a sperm cell, preference is given to sperm cell which appear morphologically normal and which swim progressively forward. The motile sperm is aspirated into the injection pipette; the pipette is lifted and moved to the clean PVP droplet. The pipette is lowered to just above the surface (in the PVP) droplet), the sperm is slowly ejected moving the pipette up or down so that the sperm's tail is transversally positioned to the pipette. The pipette point is lowered onto the section of the tail just below the midpiece. The pressure and the movement of the microinjection needle on and over the sperm tail, destabilizes the sperm membrane system and immobilizes the sperm. Destabilizing the sperm membrane before ICSI

appears to be of crucial importance for decondensation of the sperm head and pronuclei formation. It must be ensured that the sperm is immotile before injection, as a motile sperm may cause structural damage within the oocyte ooplasma. The sperm cell is aspirated and ejected repeatedly to ensure that the sperm cell does not stick to the injection pipette. The selected immobilized sperm is aspirated tail first into the microinjection pipette. The microinjection pipette containing the sperm is lifted and moved to the oocyte droplet.

For testis biopsied sperm

The number of sperm obtained after the separation process is extremely low. The resuspended pellet obtained from the gradient centrifugation must therefore be used to prepare a Petri dish as shown in the diagram above. Multiple sperm drops are placed directly into the injection dish as well as few very small sperm prep drops. The configuration of the PVP and oocyte injection drops is the same as for ICSI with ejaculated sperm. After preparation the dish is placed into an incubator for a few minutes to allow all the cells to settle. The Petri dish is placed on the heated stage and the droplets searched for motile spermatozoa. Motile spermatozoa found in the droplets are aspirated with a special testis biopsy pipette and transferred to the small medium droplets, until a sufficient number has been obtained. The spermatozoa are then directly transferred to the PVP droplet of the microinjection Petri dish for immobilization and injection.

Oocyte microinjection

Using the microinjection pipette the oocyte is rotated to locate the polar body at the 12 o'clock or 6 o'clock position. The holding pipette is lowered and the oocyte held by gentle suction. The oocyte is lowered so that the oocyte touches the bottom, as this provides better control during injection. The microscope is focused on the oocyte's equatorial plane and the internal lumen of the holding pipette. The microinjection pipette is lowered into the same focus plane at the 3 o'clock position. The plane of the microinjection pipette can be corrected by gently pushing on the zona pellucida. The sperm cell is carefully brought forward to the point of the microinjection pipette and the pipette pushed carefully through the zona pellucida and through the oolemma into the ooplasma. The successful penetration of the oolemma is indicated by the ability to aspirate ooplasma into the pipette. The ooplasma and the sperm cell are then carefully ejected into the oocyte and the microinjection pipette withdrawn and the oocyte is released from the holding pipette. The process of penetrating the oolemma and the aspiration of a small volume of ooplasma also helps to activate the oocyte essential for the normal progress of fertilization. Care must be taken not to eject a large volume of PVP medium into the oocyte, because this

inevitably leads to oocyte degeneration. This whole procedure is repeated for all the oocytes in the microinjection dish.

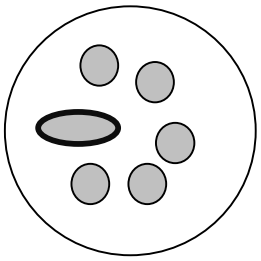
The oolemma of oocytes may have differing elasticity depending on the hyper stimulation regiment followed, the female patient's age, the maturation of the oocyte and the in vitro age. A highly elastic oolemma makes penetration difficult (deeply invaginates when penetration is attempted). Repeat penetration may therefore be required to successfully penetrate the oolemma. These repeat penetrations must be performed without penetrating the opposite oolemma, as this may result in oocyte degeneration.

APPENDIX III Drs Aevitas Fertility Unit: Embryo Culture & Evaluation

Embryo evaluation

Dish preparation for embryo culture

- Prepare a culture dish with cleavage medium drops (Quinns – Cooper Surgical) for the next day
- Make drops under oil (Quinns – Cooper Surgical)
    - Work as fast as possible to prevent evaporation
    - Work on a cold surface
    - Make drops equal to the number of oocytes (but add one extra for rinsing)
    - Maximum number of drops is 10 – make 2 dishes if more than 10



For 6 embryos

Fertilization check

Day 1 (2 PN)

- Check for pro nuclei (PN) and polar body (PB) number on the inverted microscope with heated stage
    - Note any abnormal number of PN [> or < than 2], or any other anomalies
- Transfer to preincubated cleavage medium drops covered with mineral oil in a small Petri dish
- Incubate overnight at 37 ºC, 6% CO_2

Embryo quality/morphology check

Day 2 (2- 4 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation*
- Select embryos for transfer if a day 2 transfer
    - *See section on embryo transfer*

Day 3 (6-8 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation*
- Transfer embryos to preincubated blastocyst medium drops covered with mineral oil
- Select embryos for transfer if a day 3 transfer
    - *See section on embryo transfer*

Day 5/6 (Blastocyst transfer)

- Check for cell stage/blastocyst morphology on the inverted microscope with heated stage
    - *See section on blastocyst morphology evaluation*
- Transfer embryos/blastocysts to preincubated blastocyst medium drops covered with mineral oil
- Select embryos/blastocysts for transfer if a day 5 transfer
    - *See section on embryo transfer*

Cryopreservation

Select blastocysts for vitrification after transfers

- *See section on blastocyst vitrification*

APPENDIX IV Grading Criteria: Good Quality Embryos

Embryo Grading (Good Quality Criteria)		
	Cell	**Grading**
Day 2	2 Cell 3 Cell 4 Cell	4 5
Day 3	6 Cell 7 Cell 8 Cell	4 5
Day 4	10 Cell Early Compact (EC) Compact	4 5
Day 5	Blastocyst 1 B or A	1, 2, 3 B or A

APPENDIX V Modified Gardner & Schoolcraft Blastocyst Grading System

Degree of Expansion & Hatching Status	
1	Early blastocyst; the blastocoel filling more than half the volume of the conceptus, but no expansion in overall size as compared to earlier stages.
2	Blastocyst; the blastocoel filling more than half of the volume of the conceptus, with slight expansion in overall size and notable thinning of the zona pellucida.
3	Full blastocyst; a blastocoel more than 50% of the conceptus volume and overall size fully enlarged with a very thin zona pellucida.
4	Hatching blastocyst; non-preimplantation genetic diagnosis. The trophectoderm has started to herniate through the zona.
5	Fully hatched blastocyst; non-preimplantation genetic diagnosis. Free blastocyst fully removed from zona pellucida.
6	Hatching or hatched blastocyst; preimplantation genetic diagnosis.

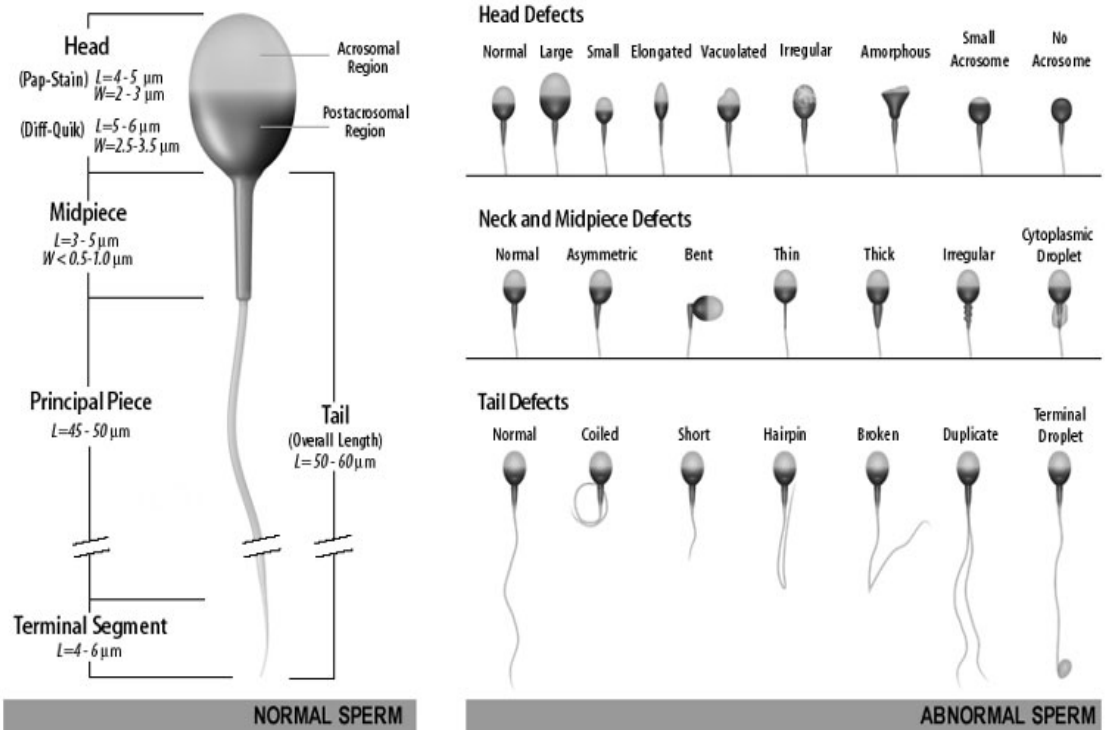
Inner Cell Mass (ICM) Grading	
A	Tightly packed, compacted cells
B	Larger, loose cells
C	No distinguishable ICM
D	Cells of the ICM appear degenerative

Trophectoderm Grading	
A	Many healthy cells forming a cohesive epithelium
B	Few, but healthy cells, large in size
C	Poor, very large, or unevenly distributed cells; may appear as few cells squeezed to the side
D	Cells of the trophectoderm appear degenerative

APPENDIX VI Sperm morphology (WHO)

Spermatozoa consist of a head, middle piece (mid-piece), principal piece and end-piece. As the end-piece is difficult to see with a light microscope, the cell can be considered to comprise a head (and a neck) and tail (mid-piece and principal piece). Both the head and tail must be free from abnormalities for a spermatozoon to be considered normal. All borderline forms should be considered abnormal.

- The head should be smooth, regularly contoured and generally oval in shape. There should be a well-defined acrosomal region comprising 40 – 70% of the head area (Menkveld *et al.*, 2001). The acrosomal region should contain no large vacuoles, and not occupy more than 20% of the sperm head. The post-acrosomal region should not contain any vacuoles.
- The mid-piece should be slender, regular and about the same length as the sperm head. The major axis of the mid-piece should be aligned with the major axis of the sperm head. Residual cytoplasm is considered an anomaly only when in excess, i.e. when it exceeds one third of the sperm head size (Mortimer & Menkveld, 2001).
- The principal piece should have a uniform calibre along its length, be thinner along the mid-piece, and be approximately 45µm long (about 10 times the head length). It may be looped back on itself, provided there is no sharp angle indicative of a flagellar break.



APPENDIX VII Standard Protocol: PICSI® Procedure

SOP 11: Physiological Intracytoplasmic sperm injection – PICSI® (Tygerberg Fertility Clinic)

PICSI® procedure

Make sure that all forms and documents are prepared
Check patient's file and record to eliminate all possible uncertainties/queries

Semen preparation, oocyte aspiration, denuding of oocytes, injection procedure/technique, embryo evaluation, embryo transfer and cryopreservation is exactly as for ICSI **[SOP 10]**

PICSI®® dish preparation for use

- Hydrate the hyaluronan microdots by placing single 10-µL elliptical droplets of Sperm preparation medium [SAGE] at the end of each locating line covering the area where the microdot is situated

- Also add a drop polyvinylpyrrolidone (PVP) and HEPES buffered drop and carefully flood the dish with tissue culture oil

- Leave for ± 5minutes and add a small volume [10µL] of prepared sperm to the drop

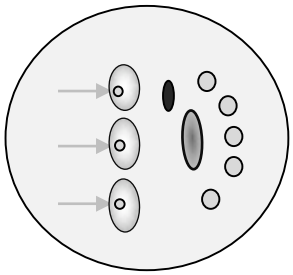
    - Touch the tip of the micropipette containing the sperm to the edge of the hydrating drop at the bottom of the dish under the oil and expel the sperm
    - By delivering the sperm in a volume equal to the hydrating fluid, immediate mixing and delivery of sperm to the vicinity of the microdot is assured
    - If the sperm are delivered in a smaller volume at the edge of the drop, greater than 30 minutes may be required for them to swim through the hydrating fluid to the microdot

- Alternatively, the sperm suspension can be added directly to the dry microdot.

- Sperm binding begin normally in 5 minutes or less

- Some microdots may require 30 minutes or more to reach full binding capability

Therefore, whenever marginal sperm binding is observed, pre-hydrate for 30minutes or more, or allow sperm to incubate on the dot for 30 minutes or more before selecting sperm



Sperm preparation medium with selected sperm cells

PVP

HEPES buffered medium

Hyaluron drop with sperm

Sperm Selection for injection

- Once bound, hyaluronan bound sperm are easily identified: they exhibit no progressive migration despite vigorous tail beating
- Factors governing sperm binding: To rapidly populate the microdot with bound sperm, place approximately 100,000 hyaluronan-binding sperm per mL (approximately 1,000-2,000 total sperm in10-20 µL volume) over the microdot

Sperm Location Selection

- The wall of the hyaluronan microdot is a physical barrier to which many sperm will bind since this is usually the first point of contact
- It is sometimes difficult to distinguish whether the sperm are bound or simply swimming against the edge of the microdot. You may be sure of selecting bound sperm by selecting them from the interior of the microdot
- Obtaining a good density of bound sperm: If the density of bound sperm is too high or too low for good sperm selection, dilute or concentrate the prepared sperm sample and use the adjusted sperm sample to seed the next microdot
- Three microdots are provided on each PICSI®® Sperm Selection Device to give a sufficient opportunity

Sperm collection

- To collect a bound sperm, position the tip of the ICSI micropipette next to the sperm and gently suck fluid into the pipette, drawing in the sperm
- Place in the small reservoir drop of sperm prep
- Continue collecting until 20-50 sperm are captured
- Aspirate a single selected sperm cell and place in the PVP and immobilize
- Proceed to do the standard ICSI injection [SOP 10]

Temperature

- Sperm bind best to hyaluronan hydrogel at temperatures below 30°C
- At temperatures above 30°C, sperm swimming vigour increases and the swimming force may overcome the binding force
- The result is that about one-third of sperm bound at room temperature will show some progressive migration at 37°C and may be deemed not bound and therefore immature
- PICSI®® Sperm Selection Device dishes placed on a 37°C heated stage will come to about 33°C and then remain at that temperature

- Therefore select bound sperm at room temperature – store in Sperm prep drop and warm to 37ºC before final injection

Technique considerations

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

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

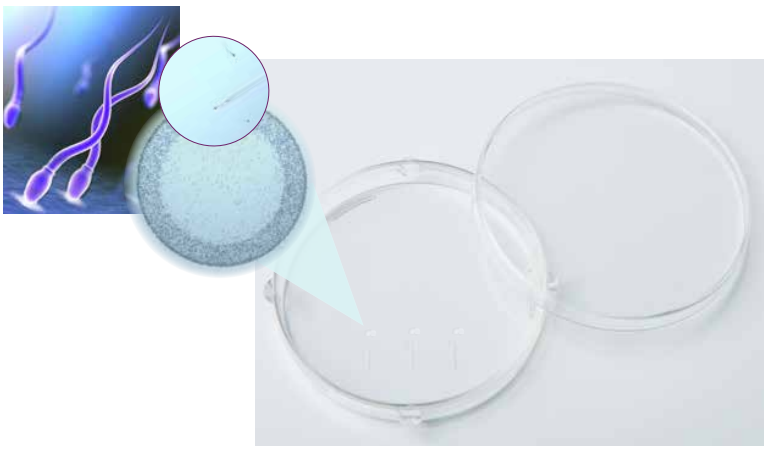
- *Microdot stability*: If a part of the wall separates from the polystyrene, the same forces that create caves can cause the microdot wall to progressively detach from the dish and coil up like a spring. When this occurs, some or all of the wall will separate from the microdot. However, the microdot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

origio

PICSI® Dish

Sperm selection for ICSI - based on Hyaluronic acid binding

- Significantly reduces pregnancy loss rate
- Binds only mature sperm with high DNA integrity
- Correlates with maturity, strict morphology and reduced chromosomal aneuploidies
- Clinically proven to benefit ICSI patients with low Hyaluronan Binding score (HBA® score)



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PICSI® Dish

Significantly reduces pregnancy Loss Rate

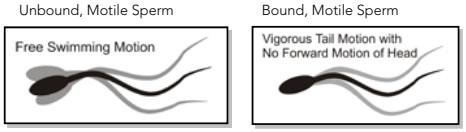
PICSI® dish is indicated for the selection of mature sperm for ICSI.

Early pregnancy loss can result from selecting a compromised spermatozoa during ICSI. This can be due to the fact that visual selection alone cannot identify mature spermatozoa with high DNA integrity and reduced chromosomal aneuploidies. Hyaluronic acid (HA)-sperm selection can.

Facts on Hyaluronan (Hyaluronic acid -HA):

- Hyaluronan is the major component of the Cumulus Complex surrounding the human oocyte
- A sperm's ability to bind to HA is a biochemical marker of the sperm's maturity and DNA integrity
- Only mature spermatozoa with developed receptors for HA can bind

The PICSI® dish contains 3 microdots of Hyaluronic acid, where mature spermatozoa will bind for easy picking.



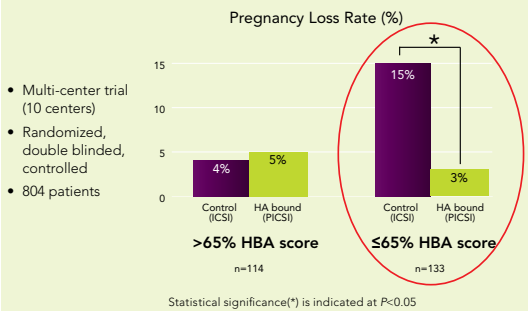
Clinical documentation

The ability to bind to HA correlates to:

- **Maturity**
- **Strict morphology**
- **High DNA integrity**
- **Reduced chromosomal aneuploidies**

In an extensive study by Worrilow et al. (2012), it was found that the combination of the diagnostic abilities of the Hyaluronic Binding Assay (HBA®) and the HA-sperm selection in the PICSI® dish led to improved clinical Pregnancy Rate (CPL) and significantly reduced Pregnancy Loss Rate in ICSI patients diagnosed to have low HA-binding ability (HBA® score ≤65%).

This study further demonstrated that 15% of all ICSI patients express sperm samples with compromised developments (HBA® score≤ 65%) and would benefit from HA sperm selection.



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Catalogue No.

BCT-PICSI-20 20 PICSI® dishes, individually packaged, sterile

Find more information on www.origio.com

A demonstration video as well as the instructions for use are availble on our website. You can also find out who your local ORIGIO distributor is.

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APPENDIX VIII Standard Protocol: IMSI Procedure

SOP 12: Intracytoplasmic Morphological Sperm Injection - IMSI

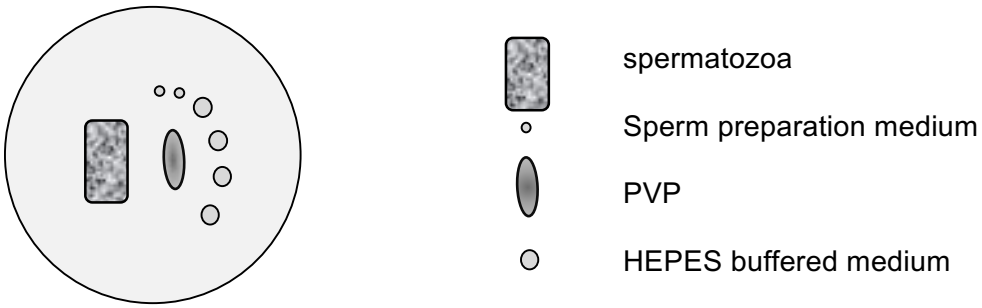
IMSI procedure

Make sure that all forms and documents are prepared
Check patient's file and record to eliminate all possible uncertainties/queries

Semen preparation, oocyte aspiration, denuding of oocytes, injection procedure/technique, embryo evaluation, embryo transfer and cryopreservation is exactly as for ICSI **[SOP 10]**

IMSI Dish preparation for use

- For IMSI with **ejaculated semen**, prepare the following injection dish (**glass bottom dish**):



- Incubate for ±30 minutes at 37ºC [no CO_2]

Sperm selection and immobilization

- Add sperm cells to the sperm preparation medium
- Select motile, morphological normal spermatozoa from the drop with the ICSI injection pipette using the 20x objective
- Place the selected sperm into the left sperm preparation drop and focus on the edge of the drop
- Change the heated stage - metal one with a hole - the dish needs to come in contact with the objective
- Put the 100x objective in place and place a small drop of oil onto the objective
- Place the glass bottom dish containing the sperm onto the oil-covered objecti ve

- The left sperm preparation drop should be in the center of the objective.
  - Use the 100X magnification to focus on the sperm preparation drop edge
  - Bring the needle down and make an indentation in the drop edge
    - The sperm will swim into the indentation
- Select morphologically normal sperm without any vacuoles and move them to the sperm preparation drop to the right
- After selecting enough sperm, change the heated stage again and proceed with the normal ICSI protocol [SOP 10]

Collect enough sperm cells for the injection procedure

APPENDIX IX Patient Information Leaflet & Consent Forms

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

"Examining The Effect of EmbryoGlue®®, Used as an Embryo Transfer Medium, on Assisted Reproductive Treatment Outcome and Success."

PRINCIPAL INVESTIGATOR: Lara Maree
ADDRESS: Drs Aevitas Fertility Clinic, Vincent Pallotti Hospital, Pinelands
CONTACT NUMBER: 082 409 8227

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

The study is to be conducted at Drs Aevitas Clinic at Vincent Pallotti Hospital. Approximately 400 couples will be included in the study. The aim of the study is to improve successful implantation of the embryo after being transferred to the patient.

EmbryoGlue®® is an embryo transfer specific medium containing a unique combination of hyaluronic acid and recombinant albumin. The hyaluron contained in EmbryoGlue®® is believed to act as a specialised adherence compound, supporting the implantation of a transferred embryo in the endometrium. A prospective randomized study in 2008 showed an

overall increase in implantation and clinical pregnancy rates where EmbryoGlue®® was favored above conventional transfer medium.

The research is being conducted to determine whether the use of EmbryoGlue®® will improve implantation rates and ongoing pregnancy rates in Drs Aevitas Clinic.

Why have you been invited to participate?

You have been identified as a suitable candidate and invited to participate in the research based on your decision to undergo infertility treatment at Drs Aevitas Clinic. You qualify as a participant due to:

- Female partner age ≤36
- Normal BMI, unobstructed fallopian tubes,
- Good response to ovarian stimulation (≥ 5 ova collected during aspiration)
- ≥ 2 good embryos to transfer

What will your responsibilities be?

All we ask is that you read through the information and sign the form provided to give consent for the principle and co-investigators involved in this study to access to your personal information and for EmbryoGlue®® to be used for your embryo transfer.

Will you benefit from taking part in this research?

You will benefit from participating in this study in knowing that you are helping to make possible research that aims to improve the outcome of successful pregnancy of sub-fertile couples, including yourself, wishing to conceive through ART in fertility units.

Are there in risks involved in your taking part in this research?

There are no additional risks associated in taking part in this research.

If you do not agree to take part, what alternatives do you have?

If you choose not to take part in the research the standard protocol for embryo transfer will be used in your treatment.

Who will have access to your medical records?

Your identity and any additional information collected through your participation will be treated as confidential and protected. If the research is used in a publication or thesis, the identity of

the participant will remain anonymous. This information will be available only to the principle and co-investigators involved in the research project described.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

The possibility of research related injury has been considered and it has been determined that it is highly unlikely that any form of injury may occur as a direct or indirect result of participation.

Will you be paid to take part in this study and are there any costs involved?

You will not be paid to take part in the study. Additional costs associated with the use of EmbryoGlue® will be covered by the research team.

Is there anything else that you should know or do?

- You can contact Dr. ML Windt de Beer at Tel: (021) 938 4851 if you have any further queries or encounter any problems.
- You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……..…… agree to take part in a research study entitled *(insert title of study)*.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2015.

....

Signature of participant **Signature of witness**

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2015.

....

Signature of investigator **Signature of witness**

APPENDIX X EmbryoGlue® Product Insert

EmbryoGlue®

3044/1.0/0

Vitrolife

EN: Indication for use:

Medium for embryo transfer.

Product Description

EmbryoGlue® is a bicarbonate buffered medium containing recombinant human albumin, hyaluronan and gentamicin as an antibacterial agent.

For use after equilibration at +37°C and 6 % CO_2 atmosphere.

Storage instructions and stability

Store dark at +2 to +8 °C.

EmbryoGlue® is stable until the expiry date shown on the container labels and the LOT-specific Certificate of Analysis.

Media bottle should not be stored after opening. Discard excess media after completion of the procedure.

Directions for use:

The following is the general procedure for performing embryo transfer using EmbryoGlue®. For more information on the recommended use of EmbryoGlue®, please see "Vitrolife G Series® Manual". The Manual is available on request from Vitrolife and may be obtained through the company's internet sites, www.vitrolife.com. Directions for use in other languages can also be found at the website.

Embryo transfer:

Add approximately 1 mL of EmbryoGlue® to the well of a 4-well 1-well dish. Add approximately 2 mL of EmbryoGlue® to the moat of the 1-well dish.

Equilibrate the dish in +37°C and 6 % CO_2 for 4–18 hours. Equilibrate the embryos to be transferred in the well containing EmbryoGlue® for a minimum of 10 minutes in a 6% CO_2 environment prior to transfer. Rinse the 1 mL non-toxic syringe by drawing up and then out media from the moat several times until no air bubbles are observed in the syringe. Draw up approximately 0.5 mL of the medium from the moat. Firmly attach the transfer catheter to the pre-rinsed 1 mL non-toxic syringe. Flush approximately 0.5–1.0 mL of equilibrated transfer medium from the moat through and out of the catheter. After rinsing, draw approximately 0.1 mL of EmbryoGlue® from the center well and expel into the moat until approximately 20 µL is left in the syringe. Under microscopic control, gently load the embryos into the catheter in approximately 5–10 µL of additional EmbryoGlue® followed by a small amount of air. (The small pocket of air at the tip allows better imaging of the tip for ultrasound guided transfers). For the embryo transfer, pass the tip of the catheter into the uterus approximately 1 cm from the top of the cavity and expel the embryos in a total volume of approximately 25–30 µL of medium. Slowly withdraw the catheter while maintaining steady pressure on the plunger of the syringe. Make a final microscopic examination of the catheter.

Specifications

Sterile filtered	SAL 10⁻³
Mouse Embryo Assay (1-cell) (% expanded blastocysts on day 5)	≥ 80
Bacterial endotoxins (LAL assay) (EU/mL)	< 0.25

LOT specific test results are available on the Certificate of Analysis provided with each delivery.

Symbols

	Sterilized using aseptic processing technique
	Temperature limitation
	Do not re-use, discard after procedure.
	Use by - see label.
	Caution: Consult accompanying documents.
	Catalog number
	Batch code

Precautions

Do not use EmbryoGlue® if it appears cloudy.

EmbryoGlue® contains recombinant human albumin, hyaluronan and gentamicin.

Vitrolife recommends that media be opened and used only with aseptic technique.

The risk of reproductive toxicity and developmental toxicity for IVF-media, including Vitrolife's IVF-media, have not been determined and are uncertain.

Not for injection.

Caution: Federal (US) law restricts this device to sale by or on the order of a physician.

DE: Anwendungshinweise:

Für den Embryotransfer.

Produktbeschreibung

EmbryoGlue® ist ein Bikarbonat-gepuffertes Medium mit rekombinantem humanem Albumin, Hyaluronsäure und Gentamicin als Antibiotikum.

Die Anwendung erfolgt nach der Equilibrierung bei +37 °C und 6 %iger CO_2-Atmosphäre.

Lagerung und Haltbarkeit

Vor Licht geschützt bei +2 bis +8 °C lagern.

EmbryoGlue® ist bis zum auf den Verpackungsetiketten und im Analysenzertifikat der Charge angegebenen Verfallsdatum haltbar.

Die Flaschen dürfen nach dem Öffnen nicht aufbewahrt werden. Ensorgen Sie medizinisches Medium nach Abschluss des Vorgangs.

Anwendung

Nachstehend wird das allgemeine Verfahren zum Embryotransfer mit EmbryoGlue® beschrieben. Weitere Informationen zur empfohlenen Anwendung von EmbryoGlue® finden Sie im „Vitrolife G Series® Handbuch". Das Handbuch ist auf Anfrage bei Vitrolife erhältlich oder kann auf der Unternehmenswebseite www.vitrolife.com bezogen werden. Die Anwendungsanweisungen in weiteren Sprachen entnehmen Sie bitte der Website.

Embryotransfer:

Geben Sie etwa 1 ml EmbryoGlue® in die Mitte einer geeigneten 1-well-Schale. Geben Sie etwa 2 ml EmbryoGlue® in die Vertiefung der 1-well-Schale. Equilibrieren Sie die Schale 4– 18 Stunden lang bei +37 °C und 6 %iger CO_2-Atmosphäre. Equilibrieren Sie auch die für den Transfer in die Schale mit EmbryoGlue® vorgesehenen Embryonen vor dem Transfer für mindestens 10 Minuten bei 6 %iger CO_2-Atmosphäre. Spülen Sie die nicht toxische 1-ml-Spritze, indem Sie so lange Medium aufziehen und wieder abgeben, bis sich in der Spritze keine Bläschen mehr bilden. Ziehen Sie etwa 0,5 ml Medium aus der Schale auf. Verbinden Sie den Transferkatheter fest mit der vorgespülten und nicht toxischen 1-ml-Spritze. Spülen Sie 0,5– 1,0 ml equilibrierten Transfermedium aus der Schale durch den Katheter. Ziehen Sie nach dem Spülen etwa 0,1 ml EmbryoGlue® aus der Centerwell-Schale auf und geben Sie es in die Vertiefung zurück, bis nur noch etwa 20 µl in der Spritze erhalten. Ziehen Sie die EmbryoGlue® unter dem Mikroskop vorsichtig mit etwa 5– 10 µl zusätzlichem EmbryoGlue® und Luft in den Katheter. (Die Luft an der Spitze macht diese auf dem Ultraschallbild besser sichtbar). Führen Sie die Spitze bis etwa 1 cm vor der Gebärmutterhöhle in den Uterus ein und setzen Sie die Embryonen mit einem Gesamtvolumen von etwa 25– 30 µl Medium ab. Ziehen Sie den Katheter anschließend vorsichtig heraus, wobei Sie den Druck auf den Kolben der Spritze erhalten. Untersuchen Sie den Katheter abschließend unter dem Mikroskop.

Produktdaten

Steril filtriert	SAL 10⁻³
Mouse Embryo Assay (1-zell) (% expandierte Blastozysten an Tag 5)	≥ 80
Bakterielle Endotoxine (LAL-Test) (EU/mL)	< 0,25

Chargen-spezifische Testergebnisse finden Sie auf dem Analysenzertifikat, das jeder Lieferung beiliegt.

Symbole:

	Unter Verwendung aseptischer Techniken sterilisiert
	Temperaturbeschränkung
	Nicht wiederverwenden, direkt nach Gebrauch entsorgen
	Verfallsdatum - siehe Etikett
	Hinweis: Lesen Sie bitte die beigefügten Unterlagen
	Katalognummer
	Chargencode

Vorsichtsmaßnahmen

Verwenden Sie EmbryoGlue® nicht, wenn es trübe erscheint.

EmbryoGlue® enthält rekombinantes humanes Albumin, Hyaluronsäure und Gentamicin.

Vitrolife empfiehlt, die Medien nur unter Anwendung von aseptischen Techniken zu öffnen und zu verwenden.

Die Risiken einer Toxizität von IVF-Medien in Hinblick auf Reproduktion und Entwicklung, inklusive der IVF-Medien von Vitrolife, wurden noch nicht bestimmt und gelten als unsicher.

Nicht zur Injektion bestimmt.

Hinweis: Nach US-amerikanischem Gesetz darf dieses Produkt nur von einem Arzt gekauft oder gegen Vorlage eines ärztlichen Rezeptes erworben werden."

ES: Instrucciones de uso

Medio de transferencia embrionaria.

Descripción del producto

EmbryoGlue® es un medio tamponado con bicarbonato que contiene albúmina humana recombinante, hialuronano y gentamicina como farmaco antibacteriano.

A utilizar después de equilibrar en una atmósfera +37 °C y con CO_2 al 6 %.

Instrucciones de conservación y estabilidad

Conservar en un lugar oscuro de +2 a +8 °C.

EmbryoGlue® es estable hasta la fecha de caducidad que aparece en el etiquetado del envase y el certificado de análisis del LOT correspondiente.

Los frascos no deben conservarse una vez abiertos. Desechar el exceso de medio después de finalizar el procedimiento.

Instrucciones de uso

A continuación se indica el procedimiento general para efectuar la transferencia embrionaria mediante EmbryoGlue®. Para más información sobre el uso recomendado de EmbryoGlue®, consulte por favor el "Manual Vitrolife de la serie G®". El manual debe solicitarse a Vitrolife, pudiendo encargarse a través de la página web de la empresa: www.vitrolife.com. En ella también pueden encontrarse instrucciones de uso en otros idiomas.

Transferencia embrionaria

Añada aproximadamente 1 ml de EmbryoGlue® a una placa de 1 solo pocillo pre-lavada enjuagada. Agregue aproximadamente 2 ml de EmbryoGlue® al foso de la placa de un solo pocillo. Equilibre la placa a +37 °C y con CO_2 al 6 % durante 4 a 18 horas. Antes de la transferencia, equilibre los embriones que van a ser transferidos en el pocillo que contiene EmbryoGlue® durante un mínimo de 10 minutos y en un ambiente de CO_2 al 6 %. Enjuague la jeringa no tóxica de 1 ml aspirando y expulsando varias veces el medio del foso de la placa hasta que no se observen burbujas de aire. Aspire aproximadamente 0,5 ml del medio del foso. Conecte bien apretado el catéter de transferencia a la jeringa no tóxica de 1 ml previamente enjuagada. Haga pasar por el catéter (dirección de 0,5 a 1 µl ml del medio de transferencia equilibrado para lavarlo) comenzó en el foso. Después de enjuagar, aspire aproximadamente 0,1 ml de EmbryoGlue® difícil el pocillo central y vierale en el foso hasta que queden aproximadamente 20 µl en la jeringa. Bajo control microscópico, introduzca los embriones cuidadosamente en el catéter con unos 5–10 µl adicionales de EmbryoGlue®, seguidos de una pequeña cantidad de aire. (La pequeña cámara de aire en la punta permite obtener imágenes mejores de la misma en las transferencias con ayuda ecográfica). Para transferir los embriones, introduzca la punta del catéter en el interior del útero a aproximadamente 1 cm de la parte superior de la cavidad y expulse los embriones en una volumen total de aproximadamente 25 a 30 µl de medio. Retire lentamente el catéter manteniendo apretado el émbolo de la jeringa. Haga un examen microscópico final del catéter.

Especificaciones

Filtrado estéril	SAL 10⁻³
Ensayo en embrión de ratón (1 célula) (% de blastocistos en particular el día 5)	≥ 80
Endotoxinas bacterianas (ensayo LAL) (UE/ml)	< 0,25

Los resultados de los ensayos específicos de cada LOT aparecen en el certificado de análisis suministrado con cada entrega.

Símbolos

	Esterilizado con técnicas asépticas
	Limitaciones de temperatura
	No reutilizar, desechar tras el procedimiento
	Usar antes de - consultar la etiqueta
	Atención: Consulte la documentación adjunta
	Número de catálogo
	Código de lote

Precauciones

No utilice EmbryoGlue® si tiene aspecto turbio.

EmbryoGlue® contiene albúmina humana recombinante, hialuronano y gentamicina.

Vitrolife recomienda que los medios se abran y utilicen solamente con técnica aséptica.

Los riesgos de toxicidad para la reproducción y el desarrollo que conllevan los medios de FIV, incluidos los de Vitrolife, no se han determinado y son inciertos.

No inyectable.

Atención: Las leyes federales de los Estados

APPENDIX XI Standard Operating Protocol: Semen Preparation

SOP 5: Semen Preparation (Tygerberg Fertility Clinic)

The sperm preparation method is determined by the quality of the sample produced, therefore the visual/microscopic analysis of the sample is extremely important. Factors that may influence the decision are; percentage motile sperm, rate of forward progression, concentration (total count) and the number of other cells in the semen sample. In addition, the assisted reproduction procedure being followed will also determine the procedure. Whereas with in vitro fertilization [IVF], gamete intrafallopian tube transfer [GIFT] and Intrauterine insemination [IUI] procedures you may need 100 000 to $1x10^6$ cells/oocyte, you only need 1 sperm/oocyte for an intracytoplasmic sperm injection [ICSI] procedure.

Two basic procedures (wash and swim-up and continuous gradient centrifugation) and modifications of these, are used for the majority of sperm preparation procedures. The standard wash and swim-up procedure, however, remains the most commonly used procedure for sperm preparation, even for ICSI. The reasons; no foreign particles are introduced into the sample, the sample is free of other cells and the percentage of motile sperm is high. Gradient centrifugation have however been shown to yield sperm with less DNA damage due to eliminating reactive oxygen species [ROS] early on in the preparation method.

The medium used for all the sperm preparation procedures is **Quinn's™ Sperm Washing Medium [SAGE]**

All tubes are labeled with the patient's surname and all lids and tubes also with a colour sticker.

The final tube should show both partners surnames and initials.

<u>Wash and swim-up [IUI and ICSI/PICSI®/IMSI]</u>

Three 15 mL round bottomed tubes are labelled correctly with the patent's surname and initials. 5 mL sperm prep medium in one of the tubes is warmed to37ºC

On the completion of liquefaction the semen sample - produced by masturbation in a accurately labelled semen container - is diluted 1:2 (semen:medium) in a test tube and centrifuged at 350 to 400xg for 10 minutes

The supernatant is aspirated after centrifugation and the pellet resuspended with 2mL of medium and re-centrifuged at 350 to 400xg for 10 minutes

After the 2nd centrifugation the supernatant is aspirated as close as possible to the pellet and the pellet then carefully overlayed with 0.5mL of medium taking care not to disturb the pellet

The test tube is placed at an approximate 45^{o} angle at 37ºC for 30 - 60 minutes

After the swim-up period the top 2/3rds of the medium with motile sperm is carefully aspirated and placed in a clean correctly labeled test tube and stored at 37ºC until used

Swim-up samples should be used within 1-hour, post preparation

When faced with a problem sample the above procedure can be modified as follows; the number of test tubes the sample is divided into can be increased with a subsequent decrease in the volume of medium overlayed on each pellet. Different test tube shapes can also be employed. In cases of very low initial concentrations the standard flat-bottomed tubes can be replaced with conical tubes

Wash and swim-up [IVF]

The procedure is exactly as for IUI/ICSI/PICSI®/IMISI, but the final 0.5mL medium for the swim-up step is replaced with equilibrated Quinn's Advantage™ mLProtein Plus Fertilization(HTF) Medium (equilibrated in a CO_2 incubator to reach a pH of 7.2). The 30-60 minute swim-up step is also done in a CO_2 incubator

Again, after the swim-up period the top 2/3rds of the medium with motile sperm is carefully aspirated and placed in a clean correctly labeled test tube and stored at 37ºC in the CO_2 incubator until used. Swim-up samples should be used within 1-hour post preparation

Swim-Up Method

- Mix semen sample well (ensure sample has liquefied)
- Dilute sample in a 1:2 ratio (semen: sperm washing medium [SAGE® test tube
- Mix sample well, centrifuge at 450 x g for 10 minutes
- Remove supernatant, leave pellet undisturbed
- Resuspend pellet in 2 mL SpermPrep®
- Centrifuge at 450 g for 10 minutes
- Repeat once more
- Remove supernatant and carefully overlay pellet with 0.5 mL SpermPrep®
- Leave to stand at 45° angle in an 37°C incubator for 30 – 60 minutes
- Aspirate the top layer of the sample, leaving pellet undisturbed
- Estimate the concentration, motility and forward progression of the processed sample
- Keep at 37°C for < 60 minutes until use

SilSelect (FertiPro) gradient centrifugation

Masturbation samples

This technique is mainly used for samples with low concentrations, poor motility, viscous samples, samples with high concentrations of other cells/debris and testis biopsy samples

A Stock solution of SilSelect (100%) is used and different gradients created by adding Quinn's™ Sperm Washing Medium [SWM]

Gradients (in 15 mL round bottomed tubes) – prepared fresh weekly.

90%: 9 mL of stock solution plus 1.0mL of SWM
70%: 7 mL of stock solution plus 3 mL of SWM
45%: 4.5 mL of stock solution plus5.5 mL of SWM

For a 3-layer gradient, the 3 solutions are carefully layered on each other in a conical tube, starting with the 90% solution at the bottom and making sure they are not mixing

The gradient is then allowed to equilibrate at 37^{o}C for 15 minutes (Figure1)

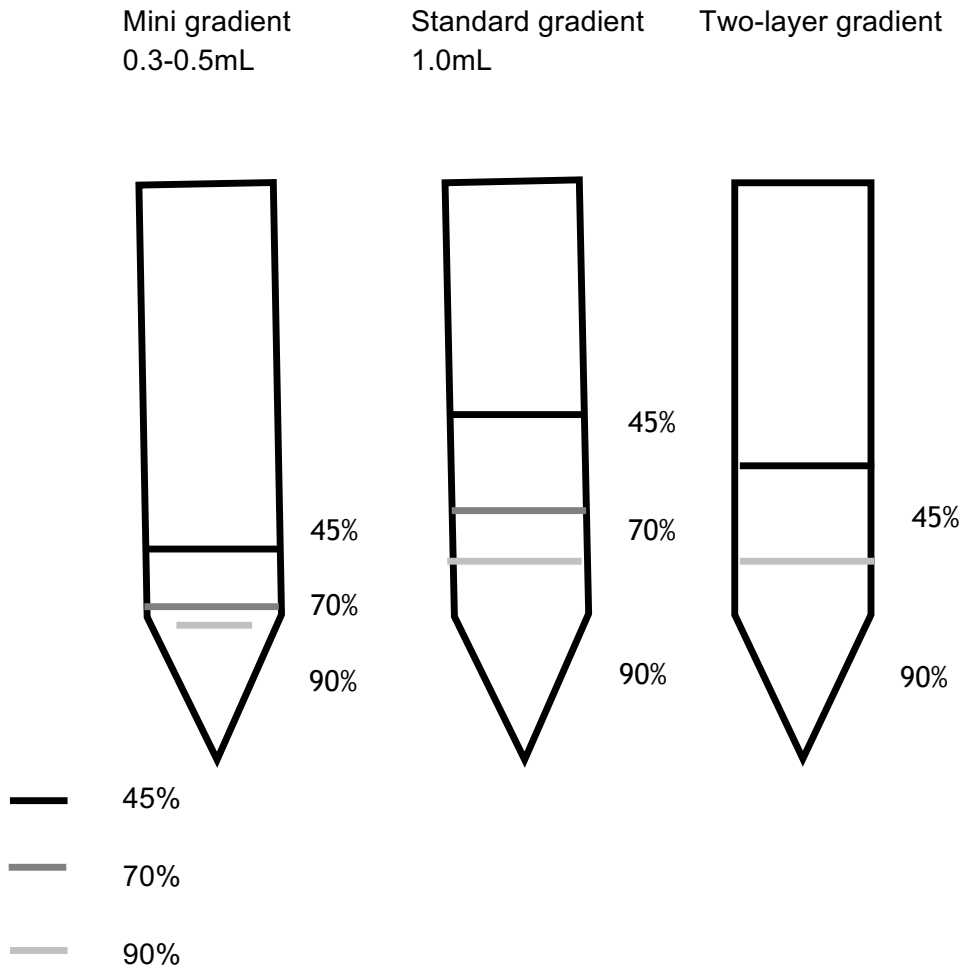
For a 2-layer gradient, the 905 and 45% is used

The volumes of the solution for the gradient depends on the fertilization procedure and the semen sample
In general I mL is used for IVF and IUI, 0.5 mL for ICSI/PICSI®/IMISI and 0.3 mL for a testis biopsy sample

The gradient should be used within 2 hours of preparation

Two 15 mL round bottomed tubes and 2 conical tubes are labelled correctly with the patent's surname and initials. 5 mL sperm prep medium in one of the round bottomed tubes is warmed to 37ºC and one of the conical tubes prepared with the gradient.

Figure 1: SilSelect gradient centrifugation



On the completion of liquefaction the semen sample - produced by masturbation in a accurately labelled semen container - is diluted 1:2 (semen:medium) in a test tube and centrifuged at 350 to 400xg for 10 minutes. Washing is performed to concentrate all possible sperm into a smaller volume t use in the gradient method. Ideally an unwashed sample should be overlaid on the gradient and can be done in samples with adequate concentration and motility.

The pellet is re-suspended in 0.5mL of medium and carefully overlaid on the gradient

The gradient is centrifuged for 15 minutes at 300xg

After centrifugation the top layer/s are carefully aspirated and the pellet with ± 0.5 mL medium remaining, placed into a clean conical tube. The pellet is then washed twice with 2 mL medium by centrifugation 9400- 450xg) for 10 minutes each.

The resulting pellet is resuspended in 0.5mL of sperm prep medium

Frozen samples [I mL final volume – 2 straws] are overlayed directly onto the gradient

When faced with a problem sample the volumes of the gradient can decreased (0.3mL; mini-gradient, Figure 1), the number of gradients can be increased and a two-step gradient can be used

The concentrations for a two-step gradient are: 90% and 45% (Figure 1). The latter procedure results in a higher final sperm concentration, but a decreased percentage of motile sperm – often used for IUI

Testis biopsy samples

The method of preparation is similar to that of masturbated semen:

An aliquot (± 0.3mL) of the fresh testis biopsy tissue/liquid containing the sperm is overlaid on the 3 layer “mini gradient” The mini gradient is three 0.3mL layers [90%, 70%, and 45% - Figure 1]. (the rest of the sample is frozen)

The gradient is then centrifuged for 20 minutes at 400g

After centrifugation the top layer/s are carefully aspirated and the pellet with ± 0.3 mL medium remaining, placed into a clean conical tube. The pellet is then washed twice with 2 mL medium by centrifugation (450xg) for 10 minutes each.

The resulting pellet is resuspended in 0.2-0.3 mL of sperm prep medium and kept at ROOM TEMPERATURE until use

It is preferable to use a thin glass pipette for aspirations to facilitate very fine and accurate removal of the supernatants

Density Gradient Centrifugation

- This technique makes use of 3 different density gradient solutions (stock solution used is SilSelect® FertiPro);
    - 90% : 9mL of stock solution plus 1.0mL SpermPrep®
    - 70% :7mL of stock solution plus 3mL SpermPrep®
    - 45% : 4.5mL of stock solution plus 5.5mL SpermPrep®
- The solutions are carefully layered on top of each other, starting with the 90% solution at the bottom of a conical tube and allowed to equilibrate at 37°C
- Mix semen sample well (ensure sample has liquefied)
- Dilute sample in a 1:2 ratio (semen: sperm washing medium [SAGE® Advantage HEPES buffered sperm preparation medium - SpermPrep®]) in a test tube or use unwashed
- Centrifuge at 350 - 400g for 10 minutes
- Resuspend pellet in 0.5 mL SpermPrep® and carefully overlay on gradient
- Centrifuge sample at 400g between 15 – 20 minutes
- Remove top gradient layers, leaving only the pellet and some medium
- Place pellet (0.3 – 0.5 mL) into a clean conical tube
- Wash twice with 2mL medium at 450 g for 10 minutes
- Resuspend in 0.3 – 0.5mL SpermPrep®

Normal sample: 1mL or 0.5 mL gradients – 2 layer or 3 layer

Testis biopsy sample: 0.3 mL gradient – 3 layer

Centrifuge for 20 minutes at 400g

HIV positive and hepatitis positive samples

On the completion of liquefaction the semen sample - produced by masturbation in a accurately labelled semen container - is diluted 1:2 (semen:medium) in a test tube and centrifuged at 350 to 400xg for 10 minutes

The pellet isresuspended in 0.5 mL sperm prep medium and overlaid on a three layer SilSelect gradient [45%, 70%, and 90%) with 90% at the bottom

The tube is centrifuged at 350-400g for 15 minutes

The 45% and 70% layer are were aspirated and the resultant pellet placed in a clean conical, test tube

The pellet is then washed twice with 2mL sperm prep medium at 400g for 10 minutes

After the second wash the resultant pellet is overlaid with 0.6-5mL sperm prep and allowed to swim up for 60 minutes at 37°C

After the swim-up period the top 2/3rds of the medium with motile sperm is carefully aspirated and placed in a clean correctly labeled test tube and stored at 37ºC in the CO_2 incubator until used

Samples should be used within 1 hour post preparation

following hour, it was not necessary to freeze the supernatant at 70°C (13, 14)

The remaining 0.7 mL was used for IUI if the results of the PCR test for HIV-1 were negative

Sep-D preparation

On the completion of liquefaction the semen sample - produced by masturbation in a accurately labelled semen container - is placed in a small petri dish

The device (Surelife SEP-D kit) contains 1mL of pre-filled semen processing medium and is `placed at 37ºC for 15 minutes to equilibrate

The cap from the tip of the device is removed and any air bubbles removed

1-1.5 mL of liquefied semen is slowly aspirated into the syringe (from a small petri dish) ensuring that no mixing of semen and mediums occurs. Keeping the device vertical is recommended

The cap is replaced and the device kept vertical without shaking and incubated at 37^{o}C for one hour

After 1 hour, the cap is removed and the semen layer gently expelled followed by culture medium retaining only 0.5mL of culture medium containing the motile sperm in the device

The last 0.5 mL is then place in a round bottomed tube and concentration, motility and forward progression estimated

The sample is kept at 37ºC until use

Very viscous samples

Viscous samples can be treated in the same way as poorly liquefied samples

Bromolaine (proteolytic enzyme)
Prepare 10 IU/mL bromelain in Dulbecco's phosphate-buffered saline
Dilute semen 1+1 (1:2) with the 10 IU/mL bromelain
Stir with a pipette tip, and incubate at 37 °C for 10 minutes
Mix the sample well before further analysis

Washing
Add an equal volume of physiological medium (e.g. Dulbecco's phosphate-buffered saline, followed by repeated pipetting

Needle
Pass the sample gently (6–10 times) through blunt gauge 18 (internal diameter 0.84 mm) or gauge 19 (internal diameter 0.69 mm) needle attached to a syringe

These treatments may affect seminal plasma biochemistry, sperm motility and sperm morphology, and their use must be recorded

APPENDIX XII Standard Operating Protocol: In Vitro Fertilization

SOP 9: In Vitro Fertilization –IVF

Make sure that all forms and documents are prepared
Check patient's file and record to eliminate all possible uncertainties/queries

Semen preparation

See section on semen preparation methods [SOP 5]

Aspiration

Medium preparation – previous day
See section on medium preparation [SOP 4]

Ovum Pick up

- Check suction pump (100-120 mmHg)
- Prepare glass polished pipettes for pick up
- Place pick-up tubes in heated block
- Place aspirated follicular fluids in heated block and examine for oocyte-cumulus complexes using a large Petri dish on a heated stage (37-40ºC) of a dissection microscope
- Determine maturity [see appendix at end of section]
    - GV, MI, MII
    - Spread complexes to visualize the first polar body (if possible)
    - Note maturity and all other features on the ova form
- Put the complexes (with as little as possible blood and medium) in the pick-up tubes (MI and MII separate)
- When done, rinse all complexes in small Petri dish with gassed fertilization medium – check number obtained
- Now transfer to 4 well NUNC dish – maturities separate and not more than 5 complexes per well
- Incubate in the CO_2 incubator until insemination
- Complete all forms

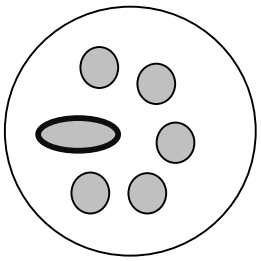
Insemination

- Inseminate complexes with the correct number/volume of prepared sperm
    - Morphology ≤ 4 % - up to 2 x 10^6 sperm/ovum
    - Morphology > 4, ≤ 14 % – 500 000 sperm/ovum
    - Morphology > 14 % – 100 000 sperm/ovum
        - *(work out the correct volume)*
- Do insemination ± 40 hours post HCG administration if at all possible
- Incubate overnight at 37 ºC, 6% CO_2

Embryo evaluation

Dish preparation for embryo culture

- Prepare a culture dish with cleavage medium drops (Quinns – Cooper Surgical) for the next day
- Make drops under oil (Quinns – Cooper Surgical)
    - Work as fast as possible to prevent evaporation
    - Work on a cold surface
    - Make drops equal to the number of oocytes (but add one extra for rinsing)
    - Maximum number of drops is 10 – make 2 dishes if more than 10



For 6 embryos

Fertilization check

Day 1 (2 PN)

- Clean oocytes with denuding pipettes (Cook Australia)
    - If oocytes are not clearly visible – dislodge first with two sterile, hypodermic needles
- Rinse well in clean 4 well NUNC dish
- Check for pro nuclei (PN) and polar body (PB) number on the inverted microscope with heated stage
    - Note any abnormal number of PN [> or < than 2], or any other anomalies

- Transfer to preincubated cleavage medium drops covered with mineral oil in a small Petri dish
- Incubate overnight at 37 ºC, 6% CO_2

Embryo quality/morphology check

Day 2 (2- 4 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation* [SOP 17]
- Select embryos for transfer if a day 2 transfer
    - *See section on embryo transfer* [SOP 18]

Day 3 (6-8 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation* [SOP 17]
- Transfer embryos to preincubated blastocyst medium drops covered with mineral oil
- Select embryos for transfer if a day 3 transfer
    - *See section on embryo transfer* [SOP 18]

Day 5/6 (Blastocyst)

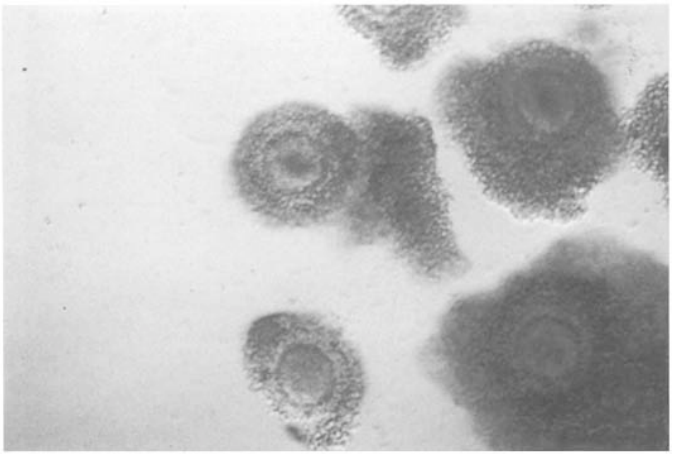
- Check for cell stage/blastocyst morphology on the inverted microscope with heated stage
    - *See section on blastocyst morphology evaluation* [SOP 17]
- Transfer embryos/blastocysts to preincubated blastocyst medium drops covered with mineral oil
- Select embryos/blastocysts for transfer if a day 5 transfer
    - *See section on embryo transfer* [SOP 18]

Cryopreservation

Select blastocysts for vitrification after transfers

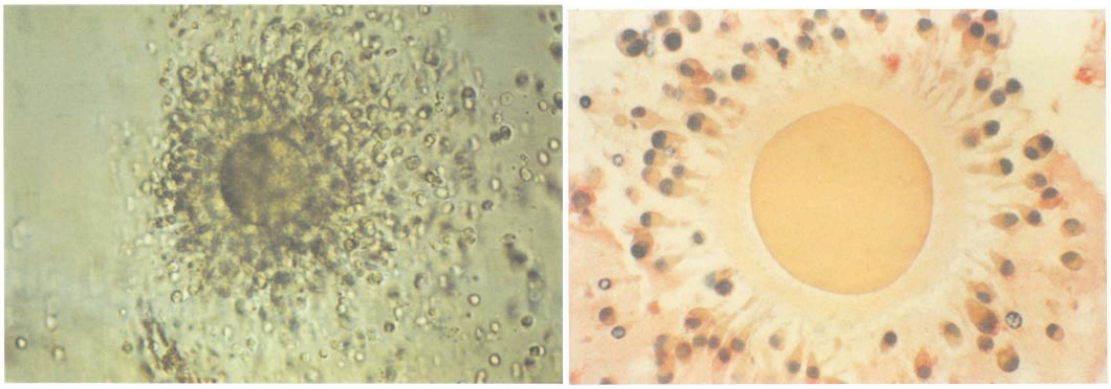
- *See section on blastocyst vitrification* [SOP13]

- **Immature COC** displays an unexpanded cumulus and a dense corona forming a compact layer of cells adhering to the zona pellucida of a prophase I (germinal vesicle-bearing) oocyte. The ooplasm cannot be seen through the cumulus mass associated with small parietal granulosa cells which appear in compact clumps

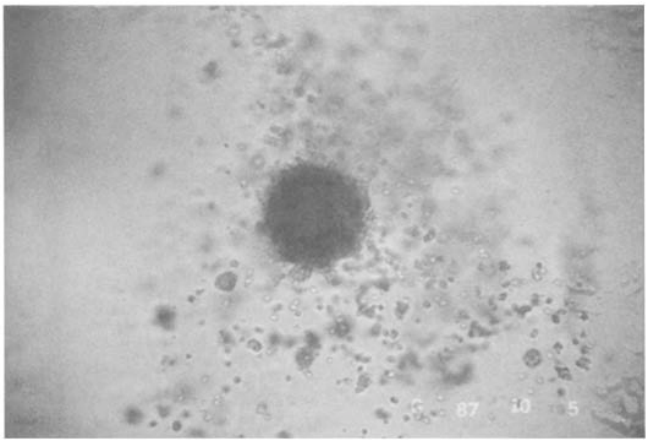


- In stimulated cycles it is common to recover COC that display some degree of cumulus expansion but a compact corona layer or even an expanded cumulus-corona complex and nevertheless contain immature prophase I oocytes. If they are not recognized at harvest, and are inseminated immediately, they will result in an absent or delayed fertilization

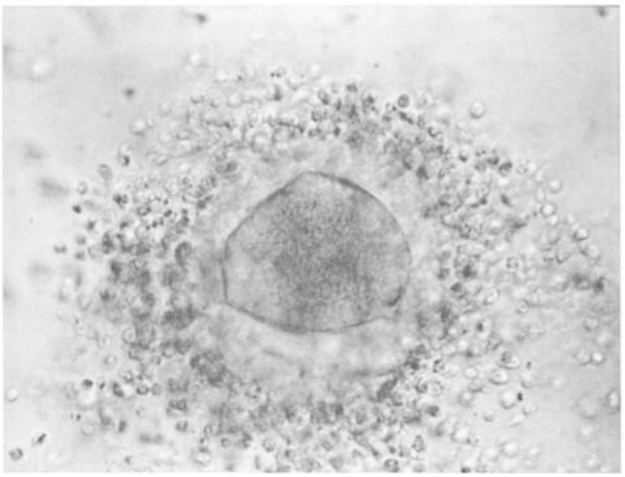
- A typical **mature preovulatory COC** displays an expanded radiating corona surrounded by the loose mass of cumulus cells, macroscopically visible. The sparse structure of these cells allows identification of the oocyte with a spherical, homogeneous ooplasm and sometimes the first polar body extruded in the perivitelline space (PVS). Usually, however, cumulus and or corona layers are dense in appearance and at times darkened and the polar body cannot be observed



- **Post-mature COC** has also been described. They are thought to arise from cycles where there has been a premature attenuated LH surge or a delayed HCG administration. The cumulus displays clusters of darkened cells while the corona is usually dark and tight



- **Degenerative or atretic COC:** At recovery, -3% of COC exhibit clear signs of anomalies, e.g. oocytes with a dark and/ or contracted irregular ooplasm, disrupted zonae pellucidae, empty zonae surrounded by a retracted cumulus mass. These COC are discarded from further culture and insemination



Jacqueline Mandelbaum. Oocytes. Human Reproduction, Vol. 15, (Suppl. 4), pp. 11-18, 2000

APPENDIX XIII Standard Operating Protocol: Vitrification & Warming-Blastocysts

SOP 13: Vitrification and Warming – Blastocysts

Blastocyst Vitrification procedure

Make sure that all forms and documents are prepared
Make sure the patients are aware of extra cost and have signed the consent form
Check patient's file and record to eliminate all possible uncertainties/queries

Modified from the Fertipro™ Vitrifreeze kit method and using the Cryotop™ from Kitazato® as carrier/storage device

Medium Preparation and labeling

- Remove an aliquot of the vitrification mediums [Fertipro™]and place into eppendorf tubes and label
    - Pre-incubation medium **[P] or 1**
    - Vitrification medium 1 **[E] or 2**
    - Vitrification medium 2 **[V] or 3**

- Allow to reach room temperature
- Use finely drawn glass pipettes
    - *Use whatever pipette and suction device you find you have the best control with*
- Label cryotops in the correct manner with a non-toxic permanent marker pen
    - Female partner surname and initials
    - DOB or Id number
    - Date of vitrification
- Complete and duplicate all applicable vitrification forms
- Find a suitable LN_2 storage place
- Check the availability of LN_2
- Get LN_2 vitri container ready
- Place 300µL of P medium in a well of a 4 well dish
- Use a big petri dish for 100 µL the medium drops of E and V respectively – make these drops just before use
- Get the LN2 ready and put the cryotop cover straws in the LN2

Artificial collapsing of blastocoel cavity

- Do artificial collapsing of expanded blastocysts – all 2 and 3 gradings of expansion
    - Use a drop of flushing medium covered with oil (37ºC)
    - Hold blastocyst with holding pipette at ICM side
    - Push collapsing pipette through trophectoderm
    - If blast does not collapse by itself, perform gentle suction to collapse blast

Method

- Place blastocysts (maximum 3) in the P medium in the w 4 well dish well.
- Leave for 5-7 minutes
    - During this time make two 50µL drops of E medium in a petri dish lid
- Set a timer for 2 minutes
- Place the blastocysts in the E medium – using as little as possible of the P medium
- Empty the pipette of all excess medium
- Move blastocysts gently around the drop to different areas
- Set a timer for 30 seconds
- After 1 minute transfer to second E drop
    - During this time make two 50µL drops of V medium in the petri dish lid
- After another 1 minute [total time in E - 2 minutes] move to the V medium drop- using as little as possible of the E medium
- Blastocysts will float – so make sure to find them and place them at the bottom of the drops
- Empty the pipette of all excess medium
- Within 30 seconds move from 1st to second V medium drop and place on Cryotop^{TM} tip in the correct manner

ALWAYS EMPTY THE PIPETTE BETWEEN TRANSFERS TO MINIMIZE DILUTION OF DROPS

- Aspirate blastocysts in a VERY small amount of V medium and place on tip of cryotop – remove most of the V medium while ensuring to keep the blastocysts on the cryotop
    - Insert the cryotop tip into the LN_2 and swirl around for a few seconds
    - Using a metal clamp to hold the cover straw, and place the tip into the cover straw – NEVER TAKE THE TIP/DEVICE OUT OF THE LN_2
- Immerge the whole device into the LN_2
- While keeping the cryotop unit under LN_2 at all times, place into a goblet and place in storage tank in the correct place

Finalize all the forms and carefully note the storage identification
Write all the details about the cryopreservation into the patient file

Vitrified blastocyst warming procedure for embyo transfer

Make sure that all forms and documents are prepared
Make sure the patients are aware of extra cost
Make sure the correct blastocysts are warmed
Make sure how many blastocysts should be warmed Check patient's file and record to eliminate all possible uncertainties/queries

Modified from the Fertipro™ Vitrithaw kit method and using the Cryotop™ from Kitazato® as carrier/storage device

Medium preparation

- Prepare a transfer dish one day before the transfer
    - 4 well dish with blastocyst medium in well 1 and 3 and blastocyst transfer medium in well 2 and 4 (37ºC, 6% CO_2)

- Remove an aliquot of the warming mediums [Fertipro™] and place into an eppendorf tube and label (to reach 37ºC)
    - Thaw medium 1 (± 1 ml in centre well dish) **[1]**
    - Thaw medium 2 **[2]**
    - Thaw medium 3 **[3]**
    - Thaw medium 4 **[4]**

- **ALL MEDIUMS MUST BE AT 37ºC**
- Use finely drawn glass pipettes
    - *Use whatever pipette and suction device you find you have the best control with*
- Check names, initials and storage place
- Use a big petri dish for the 100 µLmedium drops (Thaw 2-4)
- Get all paperwork in place

Method

- Remove CryoTop™ from the LN_2 storage tank and place into the LN_2 in container without exposing CryoTop™ to the air and take to lab
- Remove cover straw very carefully
- Insert CryoTop™ tip with embryos directly into the 1ml thaw medium 1 at 37ºC
- Stir for a few seconds and dislodge embryos with a pipette if necessary – keep this part of the step as short as possible
- Incubate the embryos **3 minutes** in thaw medium 1 (37ºC)
    - During this time make 100µL drops each of thaw solution 2,3 and 4 in a big petri dish
- Transfer the embryos to the thaw solution 2 drop and incubate for **2 minutes** (37ºC)
- Transfer the embryos to the thaw solution 3 drop and incubate for **2 minutes** (37ºC)
- Transfer the embryos to the thaw solution 4 drop and incubate for **at least 1 minute** (37ºC)

- Transfer now to ± 0.7 ml equilibrated blastocyst medium in a 4 well dish (well 1), and wash once more in the second well of the 4 well dish at 37ºC, 6% CO_2

- Note survival, expansion and blastocyst grading/quality after 2 hours
- Confirm number to be transferred
- Transfer to ET well [usually 4] just before ET

    - Fill in all applicable forms correctly

Embryo transfer is done according to SOP 20

APPENDIX XIV Standard Operating Protocol: Embryo Transfer Technique

SOP 18: Embryo Transfer Technique

Embryo Transfer Method

Preparation

- Decide which and how many embryos will be transferred after consultation with the patients and the clinician
- Transfer to the transfer dish [4 well NUNC]- prepared the previous day or 6 hours pre ET
    - 0.8 mL cleavage/blastocyst in well 2 and 3 and 2 ml medium in the middle

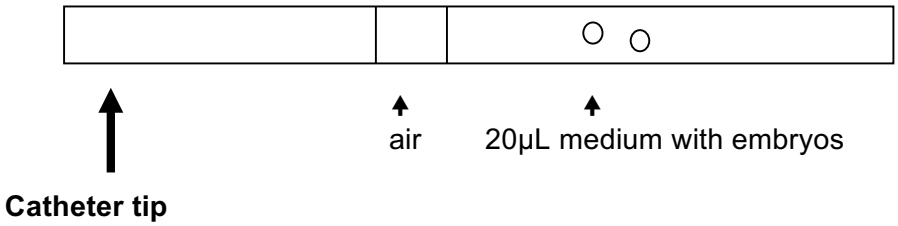
- Place sterile instruments [forceps, speculum, valsellum], sterile gauze, and the transfer catheter and stylet on a sterile green cloth
- A stylet is placed into the cannula of the soft catheter, ready for the clinician to use
- Keep 5 mL warm sterile rinsing medium ready
- The procedure and what to expect is discussed with couple and pictures of similar types of embryos also shown to them
- The patient is positioned on the bed so that she is comfortable and the clinician has good access and vision to the vagina and cervix
- The procedure starts by placing a speculum in the vagina to visualize the cervix, which is rinsed with sterile medium. The cervix is the cleaned to remove al old blood and also mucus
- It is important that the patient's bladder is full before the transfer occurs as this ensures that the endometrial cavity can be accessed easily and atraumatically and embryos transferred easily and exactly at the right place
- The procedure starts by placing a speculum in the vagina to visualize the cervix, which is rinsed with sterile medium. The cervix is the cleaned to renmoev al old blod and also mucus

Embryo Catheter loading

Once the clinician is ready for the transfer – with good sonar vision of the uterus cavity and confirmation that the cannula can be visualized in the correct place, the embryo/s are loaded into the catheter:

Method:

- Aspirate medium [blast or cleavage] from well 2 into a nontoxic 1 ml syringe
- Connect the soft catheter and expel the whole volume back inti well 2
- Make an air space of about 1 cm at the tip of the catheter
- Visualize the embryos
- Aspirate ± 10 µL of medium [± 4 cm] into the catheter and then the embryos until a total of ± 20µL in total has been aspirated
- Take to the clinician



Transfer Procedure

A soft transfer catheter, which contains the embryos to be transferred, is inserted through the cannula into uterine cavity.

After insertion of the catheter, the media containing the embryos are deposited into the uterine cavity. It is important that the embryos be placed in the correct position andthat care is taken NOT to touch the fundus

After the deposit of the embryos, the embryologist checks the catheter immediately to ensure that the embryos did not remain inside the catheter – if they did they can just be transferred againt.

The transfer is guided with an ultrasound (abdominal ultrasound) to ensure correct placement in the uterine cavity.

Anesthesia is not required when performing an embryo transfer.

It is recommended that the patient remains lying down for 15 minutes befire she gets up to empty her bladder

It is also recommended that the couple abstain from sex until the pregnancy test, the female partner avoid strenuous exercise, alcohol, caffeine, medication and cigarette smoke

Progesterone support is given until the pregnancy test – a blood 10 days after the transfer

APPENDIX XV Statistical Analysis Results (Retrospective)

Procedure Frequencies

Proc	proc3 IVF	PICSI®	ICSI	Total
1	41	0	0	41
2	0	0	129	129
3	0	29	0	29
4	0	0	52	52
5	0	14	0	14
6	0	120	0	120
8	0	0	2	2
12	0	0	1	1
Total	41	163	184	388

Age of Ova

a) Mean age of ova

proc3	variable	mean	N	sd
IVF	ageova	30.73171	41	4.636941
PICSI®	ageova	30.76687	163	4.619565
ICSI	ageova	29.91304	184	5.074639
Total	ageova	30.35825	388	4.848933

b) Mean Age of Ova Compared Between Methods

Analysis of variance (anova) used to compare the means

Number of obs = 388 R-squared = 0.0076
Root MSE = 4.84294 Adj R-squared = 0.0025

Source	Partial SS	df	MS	F	Prob>F
Model	69.405028	2	34.702514	1.48	0.2290
proc3	69.405028	2	34.702514	1.48	0.2290
Residual	9029.7986	385	23.454022		
Total	9099.2036	387	23.512154		

No difference in mean age of ova between methods p=.2290

c) PICSI®: Male Diagnosis Frequencies

Mdiag	proc3 IVF	PICSI®	ICSI	Total
1	17	36	93	146
	42.50	25.53	54.07	41.36
2	0	10	10	20
	0.00	7.09	5.81	5.67
3	0	8	1	9
	0.00	5.67	0.58	2.55
4	0	1	3	4
	0.00	0.71	1.74	1.13
5	0	3	14	17

	0.00	2.13	8.14	4.82
6	0	3	1	4
	0.00	2.13	0.58	1.13
8	0	11	19	30
	0.00	7.80	11.05	8.50
9	21	34	5	60
	52.50	24.11	2.91	17.00
10	1	1	1	3
	2.50	0.71	0.58	0.85
11	1	6	2	9
	2.50	4.26	1.16	2.55
12	0	2	21	23
	0.00	1.42	12.21	6.52
13	0	0	1	1
	0.00	0.00	0.58	0.28
15	0	24	1	25
	0.00	17.02	0.58	7.08
16	0	1	0	1
	0.00	0.71	0.00	0.28
20	0	1	0	1
	0.00	0.71	0.00	0.28
Total	40	141	172	353
	100.00	100.00	100.00	100.00

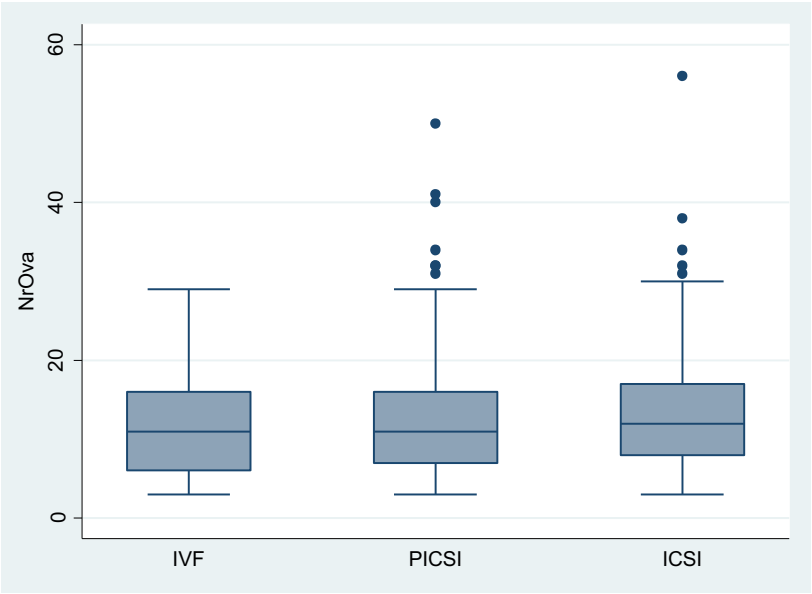
Pearson chi2(14) = 103.2437 Pr = 0.000 for comparison of PISCI versus ICSI profile.

(descriptive statistics)

d) PICSI®: Female Diagnosis Frequencies

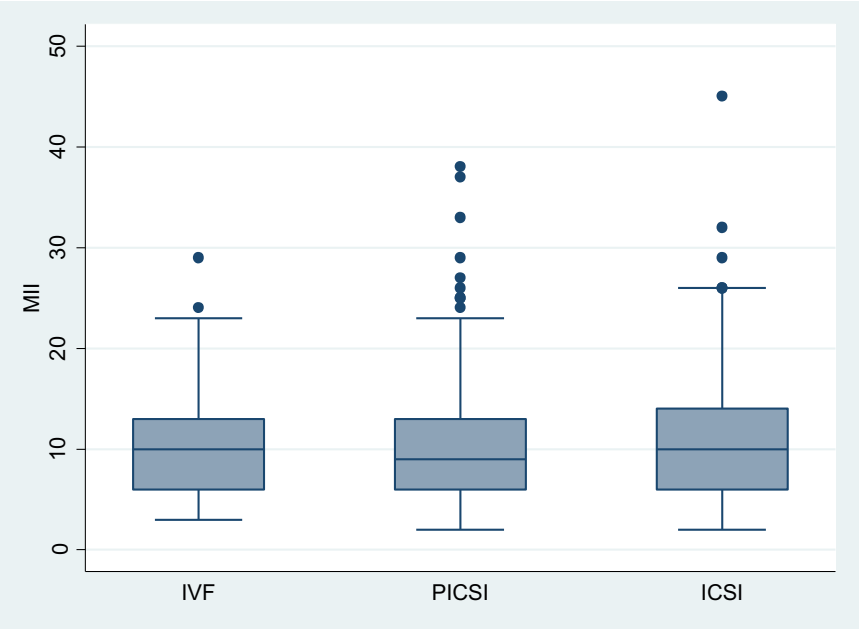
	proc3			
Fdiag	IVF	PICSI®	ICSI	Total
1	17	65	137	219
	42.50	46.43	74.86	60.33
2	6	5	9	20
	15.00	3.57	4.92	5.51
13	0	3	1	4
	0.00	2.14	0.55	1.10
14	4	12	6	22
	10.00	8.57	3.28	6.06
15	5	12	14	31
	12.50	8.57	7.65	8.54
16	6	36	13	55
	15.00	25.71	7.10	15.15
17	2	5	1	8
	5.00	3.57	0.55	2.20
18	0	1	0	1
	0.00	0.71	0.00	0.28
19	0	1	1	2
	0.00	0.71	0.55	0.55
28	0	0	1	1
	0.00	0.00	0.55	0.28
Total	40	140	183	363

e) Number of Ova



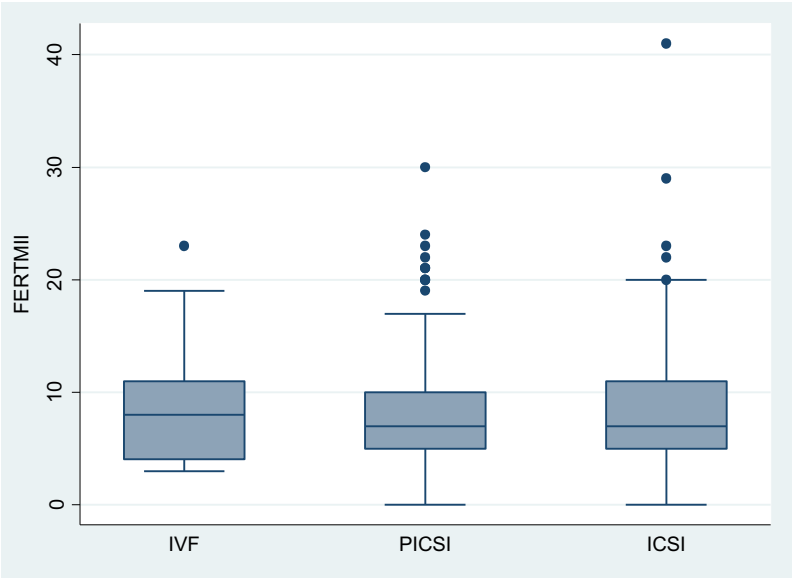

```
. ANOVA NrOva

                          Number of obs =        387    R-squared     =  0.0055
                          Root MSE      =    7.59128    Adj R-squared =  0.0004

                  Source | Partial SS          df          MS         F    Prob>F
              -----------+----------------------------------------------------
                   Model |  123.29723           2    61.648613      1.07  0.3441
                         |
                   proc3 |  123.29723           2    61.648613      1.07  0.3441
                         |
                Residual |  22128.982         384    57.627557
              -----------+----------------------------------------------------
                   Total |  22252.279         386    57.648391


. tabstat NrOva , statistics( mean sd count median ) by(proc3) varwidth(14) columns(stati
> stics)

proc3         variable |      mean        sd         N       p50
----------------------+------------------------------------------
IVF              NrOva |  11.90244  7.265689        41        11
PICSI®           NrOva |   12.8589   7.649641       163        11
ICSI             NrOva |  13.65027  7.609139       183        12
----------------------+------------------------------------------
Total            NrOva |  13.13178  7.592654       387        11
--------------------------------------------------------------------
```

f) Number of MII Ova



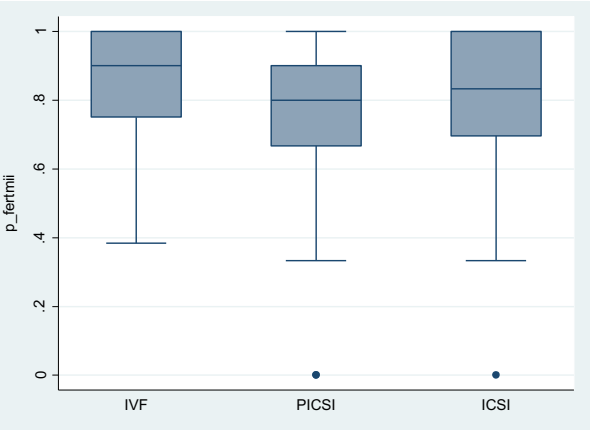

```
. ANOVA mii

                          Number of obs =        388    R-squared     =  0.0003
                          Root MSE      =    6.38634    Adj R-squared = -0.0048

                  Source | Partial SS         df         MS        F     Prob>F
              -----------+----------------------------------------------------
                   Model |  5.4036589          2   2.7018295      0.07  0.9359
                         |
                   proc3 |  5.4036589          2   2.7018295      0.07  0.9359
                         |
                Residual |  15702.377        385   40.785396
              -----------+----------------------------------------------------
                   Total |  15707.781        387   40.588581

. tabstat mii , statistics( mean sd count median ) by(proc3) varwidth(14) columns(statist
> ics)

proc3        variable |      mean        sd         N       p50
---------------------+--------------------------------------------
IVF               mii |  10.56098  6.241189        41        10
PICSI®             mii |   10.8773  6.599451       163         9
ICSI              mii |  10.96196  6.223845       184        10
---------------------+--------------------------------------------
Total             mii |  10.88402  6.370917       388        10
```

g) Number of MII Ova Fertilized




```
. ANOVA fertmii

                          Number of obs =        388    R-squared     =  0.0020
                          Root MSE      =    5.07117    Adj R-squared = -0.0032

                  Source | Partial SS         df         MS        F     Prob>F
              -----------+----------------------------------------------------
                   Model |  19.455913          2   9.7279567      0.38  0.6853
                         |
                   proc3 |  19.455913          2   9.7279567      0.38  0.6853
                         |
                Residual |  9900.9642        385    25.71679
              -----------+----------------------------------------------------
                   Total |  9920.4201        387    25.63416


. tabstat fertmii , statistics( mean sd count median ) by(proc3) varwidth(14) columns(sta
> tistics)

proc3        variable |      mean        sd         N       p50
---------------------+--------------------------------------------
IVF           fertmii |  8.585366  5.049632        41         8
PICSI®         fertmii |  8.282209  5.030167       163         7
ICSI          fertmii |  8.755435  5.111861       184         7
---------------------+--------------------------------------------
Total         fertmii |   8.53866  5.063019       388         7
```

h) Fertilization Rate of MII Ova




```
. ANOVA p_fertmii

                          Number of obs =        388    R-squared     =  0.0130
                          Root MSE      =    .176816    Adj R-squared =  0.0079

                  Source | Partial SS         df         MS        F    Prob>F
              -----------+----------------------------------------------------
                   Model |  .15902647          2   .07951324      2.54  0.0799
                         |
                   proc3 |  .15902647          2   .07951324      2.54  0.0799
                         |
                Residual |  12.036554        385   .03126378
              -----------+----------------------------------------------------
                   Total |  12.195581        387   .03151313
```

Some indication

```
. tabstat p_fertmii , statistics( mean sd count median ) by(proc3) varwidth(14) columns(s
> tatistics)

proc3        variable |      mean        sd         N       p50(median)
----------------------+-----------------------------------------
IVF          p_fertmii |  .8372943  .1753909        41        .9
PICSI®       p_fertmii |  .7741294   .180331       163        .8
ICSI         p_fertmii |  .8039342  .1739599       184  .8333333
----------------------+-----------------------------------------
Total        p_fertmii |  .7949383  .1775194       388  .8181818
```

Non-parametric regression model to compare the procedure. Quantile regression model used which models the medians. Adjusted for covariate; age of ova, donor ova and surrogate status

```
. xi: qreg p_fertmii i.proc3 ageova i.donova i.surr, quantile(50)
i.proc3           _Iproc3_0-2  (naturally coded; _Iproc3_0 omitted) (IVF the reference)
i.donova          _Idonova_1-2        (_Idonova_1 for donova==0 omitted)
i.surr            _Isurr_0-1          (naturally coded; _Isurr_0 omitted)
Iteration  1:  WLS sum of weighted deviations =  26.838704

Median regression                                 Number of obs =        387
  Raw sum of deviations 26.97327 (about .81818181)
  Min sum of deviations 26.68316                  Pseudo R2     =     0.0108

------------------------------------------------------------------------------
   p_fertmii |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Iproc3_1 |  -.0923273   .0422314    -2.19   0.029    -.1753632   -.0092915 PICSI® vs IVF
   _Iproc3_2 |  -.0605286   .0417895    -1.45   0.148    -.1426955    .0216384 ICSI vs IVF
      ageova |  -.0015345   .0036046    -0.43   0.671     -.008622     .005553
  _Idonova_2 |  -.0271952   .0389234    -0.70   0.485    -.1037267    .0493363
    _Isurr_1 |    .047144   .0791453     0.60   0.552    -.1084722    .2027602
       _cons |   .9352941   .1226313     7.63   0.000     .6941752    1.176413
------------------------------------------------------------------------------
The estimated median difference in fertilisation between PICSI® and IVF is 9.2% (95%CI: .9% to
17.5%) with PICSI® lower then IVF.  p=.029
```

The estimated median difference in fertilisation between ICSI and IVF is 6.1% (95%CI: -2.2% to 14.3%) with ICSI lower then IVF , p=.148
These contrast interesting but not the main focus of your study

Testing whether median PICSI®= median of icsi

```
. test _Iproc3_1= _Iproc3_2

 ( 1)  _Iproc3_1 - _Iproc3_2 = 0

       F(  1,   381) =    1.47
            Prob > F =    0.2258
```

Estimated difference in median fertilisation from the model between PCSI and ICSI

```
. lincom _Iproc3_1- _Iproc3_2 (PICSI®-ICSI)

 ( 1)  _Iproc3_1 - _Iproc3_2 = 0

------------------------------------------------------------------------------
   p_fertmii |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -.0317988   .0262103    -1.21   0.226    -.0833337    .0197361
------------------------------------------------------------------------------
```

Medians are not different p=.226

Testing for overall procedure effect:

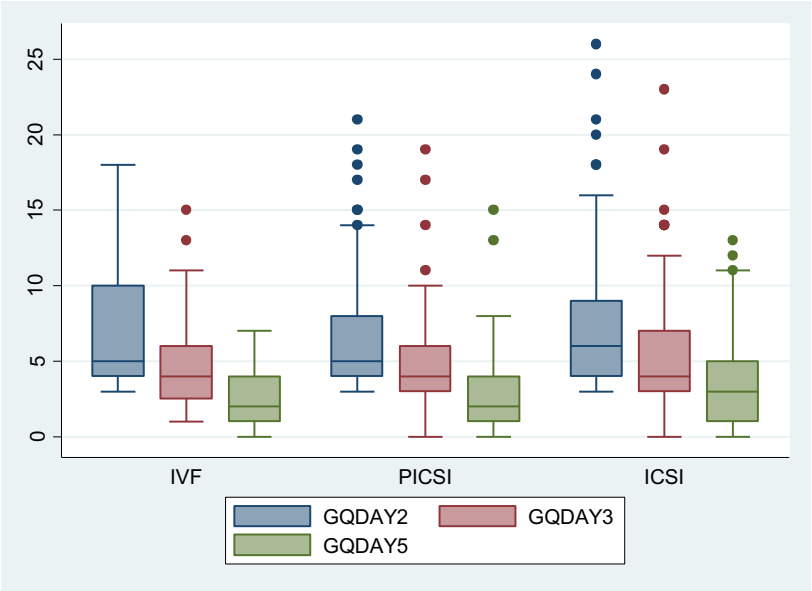
```
. test _Iproc3_1 _Iproc3_2

 ( 1)  _Iproc3_1 = 0
 ( 2)  _Iproc3_2 = 0

       F(  2,   381) =    2.53
            Prob > F =    0.0811
```

Overall there is no procedure effect. p-value similar tp that of anova above which is also an overall tests (.0811 vs .0799)

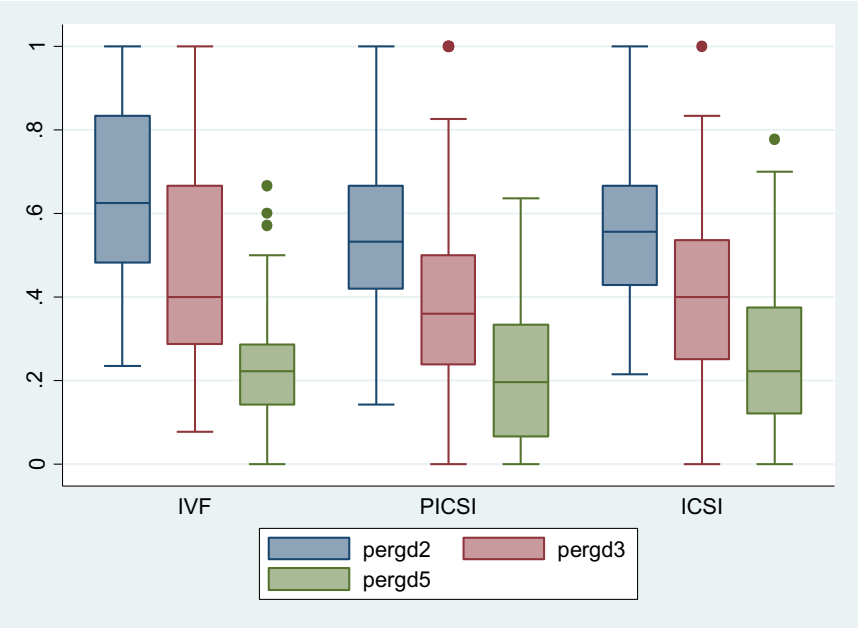
Thus no real difference between PICSI® and ICSI with respect to fertilisation of mii

i) Embryo Quality

Day2 to Day 5: Absolute number of good embryos




```
Good Fertilisation rate of number of ova over the days
. generate pergd2= GQDAY2/ NrOva
(10 missing values generated)

. generate pergd3= GQDAY3/ NrOva
(12 missing values generated)

. generate pergd5= GQDAY5/ NrOva
(104 missing values generated)

. tabstat pergd2 pergd3 pergd5 , statistics( mean sd count median ) by(proc3) varwidth(14
> ) columns(statistics)

proc3        variable |      mean        sd         N       p50
---------------------+------------------------------------------
IVF             pergd2 |  .6544362  .2257433        41      .625
                pergd3 |  .4649209  .2460165        40        .4
                pergd5 |  .2396426  .1777277        29  .2222222
---------------------+------------------------------------------
PICSI®           pergd2 |  .5403402   .171408       160   .532563
                pergd3 |  .3881474   .207578       160  .3603896
                pergd5 |  .2148705  .1670671       120  .1961538
---------------------+------------------------------------------
ICSI            pergd2 |  .5600827  .1893657       177  .5555556
                pergd3 |  .3998904  .1987751       176        .4
                pergd5 |   .248487  .1760843       135  .2222222
---------------------+------------------------------------------
Total           pergd2 |  .5619602  .1888624       378  .5454546
                pergd3 |  .4018115  .2085882       376  .3846154
                pergd5 |  .2333797    .17263       284  .2163743
-------------------------------------------------------------------
```



Profile of PICSI® and ICSI nearly identical

j) Comparison over each date between PICSI® and ICSI: Day2

```
. ttest pergd2 if proc3>0, by(proc3)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   PICSI® |     160    .5403402     .013551    .171408    .513577    .5671033
    ICSI |     177    .5600827    .0142336   .1893657   .5319922    .5881732
---------+--------------------------------------------------------------------
combined |     337    .5507094    .0098632   .1810645    .531308    .5701108
---------+--------------------------------------------------------------------
    diff |           -.0197425    .0197516              -.0585953    .0191103
------------------------------------------------------------------------------
    diff = mean(PICSI®) - mean(ICSI)                              t =  -0.9995
Ho: diff = 0                                     degrees of freedom =      335

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1591         Pr(|T| > |t|) = 0.3183          Pr(T > t) = 0.8409
```

k) Comparison over each date between PICSI® and ICSI: Day3

```
. ttest pergd3 if proc3>0, by(proc3)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   PICSI® |     160    .3881474    .0164105    .207578    .3557367     .420558
    ICSI |     176    .3998904    .0149832   .1987751   .3703193    .4294615
---------+--------------------------------------------------------------------
combined |     336    .3942985    .0110634   .2027952    .372536    .4160609
---------+--------------------------------------------------------------------
    diff |           -.0117431    .0221757              -.0553648    .0318786
------------------------------------------------------------------------------
    diff = mean(PICSI®) - mean(ICSI)                              t =  -0.5295
Ho: diff = 0                                     degrees of freedom =      334

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.2984         Pr(|T| > |t|) = 0.5968          Pr(T > t) = 0.7016
```

l) Comparison over each date between PICSI® and ICSI: Day5

```
. ttest pergd5 if proc3>0, by(proc3)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   PICSI® |     120    .2148705    .0152511    .1670671    .1846719    .2450692
    ICSI |     135     .248487    .0151549    .1760843    .2185133    .2784608
---------+--------------------------------------------------------------------
combined |     255    .2326675    .0107952     .172385    .2114081    .2539269
---------+--------------------------------------------------------------------
    diff |           -.0336165    .0215672               -.0760906    .0088575
------------------------------------------------------------------------------
    diff = mean(PICSI®) - mean(ICSI)                              t =  -1.5587
Ho: diff = 0                                     degrees of freedom =      253

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0602         Pr(|T| > |t|) = 0.1203          Pr(T > t) = 0.9398

No significant difference at all days
```

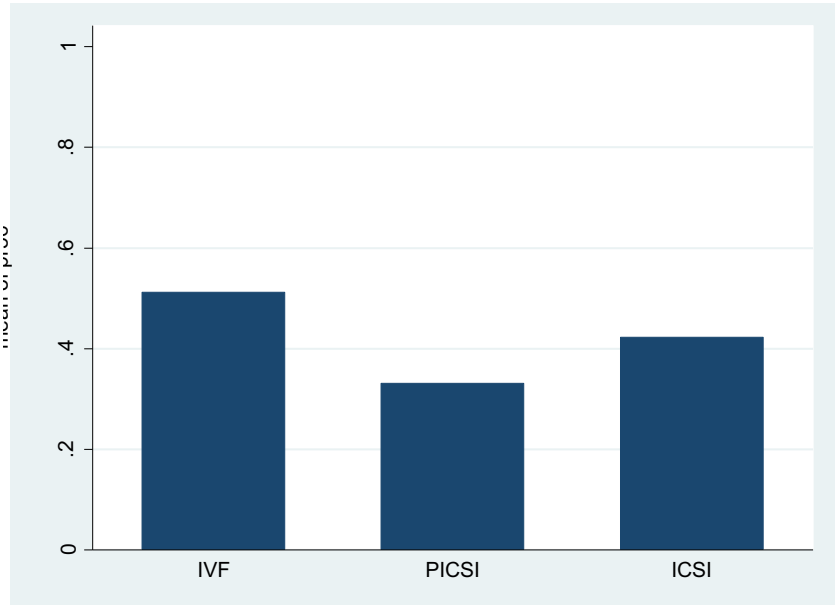
m) Pregnancy

PR	proc3 IVF	PICSI®	ICSI	Total
0	18	66	66	150
	43.90	43.71	36.67	40.32
1	0	12	17	29
	0.00	7.95	9.44	7.80
2	0	0	1	1
	0.00	0.00	0.56	0.27
3	0	0	1	1
	0.00	0.00	0.56	0.27
4	0	0	3	3
	0.00	0.00	1.67	0.81
5	4	13	3	20
	9.76	8.61	1.67	5.38
6	17	37	73	127
	41.46	24.50	40.56	34.14
7	0	6	2	8
	0.00	3.97	1.11	2.15
8	1	6	6	13
	2.44	3.97	3.33	3.49
9	0	2	1	3
	0.00	1.32	0.56	0.81
31	0	4	2	6
	0.00	2.65	1.11	1.61
32	0	4	4	8
	0.00	2.65	2.22	2.15
33	1	1	1	3
	2.44	0.66	0.56	0.81
Total	41	151	180	372
	100.00	100.00	100.00	100.00

```
. tabulate proc3 pr56, chi2 exact row
```

proc3	pr56 0	pr56 1	Total
IVF	20	21	41
	48.78	51.22	100.00
PICSI®	101	50	151
	66.89	33.11	100.00
ICSI	104	76	180
	57.78	42.22	100.00
Total	225	147	372
	60.48	39.52	100.00

Pearson chi2(2) = 5.4917 Pr = 0.064
Fisher's exact = 0.064



Binomial model of pregnancy outcome on procedure adjusted for; age of ova, donor status, number of mii and surrogate status. Risk ratios estimated. IVF is the reference category in the model.

```
. xi: binreg pr56 i.proc3 ageova i.donova mii i.surr, rr
i.proc3           _Iproc3_0-2         (naturally coded; _Iproc3_0 omitted)
i.donova          _Idonova_1-2        (_Idonova_1 for donova==0 omitted)
i.surr            _Isurr_0-1          (naturally coded; _Isurr_0 omitted)

Generalized linear models                         No. of obs      =        371
Optimization     : MQL Fisher scoring             Residual df     =        364
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  483.2354669                   (1/df) Deviance =    1.32757
Pearson          =  371.0150968                   (1/df) Pearson  =   1.019272

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u)                   [Log]

                                                  BIC             =  -1670.262

------------------------------------------------------------------------------
             |                 EIM
        pr56 | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Iproc3_1 |   .6634862   .1242177    -2.19   0.028     .4596954    .9576209 PICSI® vs IVF
   _Iproc3_2 |   .8094789   .1391561    -1.23   0.219     .5779324    1.133794 ICSI vs IVF
     ageova |   1.007773   .0191153     0.41   0.683     .9709953    1.045943
  _Idonova_2 |   1.429199   .2691808     1.90   0.058     .9880391    2.067337
         mii |   1.018637   .0088676     2.12   0.034     1.001404    1.036166
    _Isurr_1 |   .9181965   .3506057    -0.22   0.823     .4344216    1.940706
```

```
       _cons |   .2945997   .1949929    -1.85   0.065     .0805062    1.078041
------------------------------------------------------------------------------

Test for overall effect of method:

. test _Iproc3_1 _Iproc3_2

 ( 1)  _Iproc3_1 = 0
 ( 2)  _Iproc3_2 = 0

           chi2(  2) =    4.96
         Prob > chi2 =    0.0838  no difference

Testign whether PICSI® is different from ICSI:

. test _Iproc3_1= _Iproc3_2

 ( 1)  _Iproc3_1 - _Iproc3_2 = 0

           chi2(  1) =    1.97
         Prob > chi2 =    0.1605    no difference

Estimating the risk ratio beween PICSI® and ICSI:


. lincom _Iproc3_1- _Iproc3_2, rr

 ( 1)  _Iproc3_1 - _Iproc3_2 = 0

------------------------------------------------------------------------------
        pr56 |        RRR   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |    .819646   .1161671    -1.40   0.161     .6208503    1.082096
------------------------------------------------------------------------------
Risk ratio of PICSI® relative to ICSI for ongoing/full pregnancy outcome is .82 (95%CI .62 to
1.08), p=.161

PICSI® has lower pregnancy outcome rate but from this data we cannot say that it is different
from ICSI.  Sample size of the study is probably too small.

The crude unadjusted risk ratio from the table above is rr(crude)=.33/.42=.78. Thus the
adjustment for covariates  'reduces' the rr to become closer to 1 the value for the risk ratio
where pregnancy rate are the same.
```

n) Miscarriage

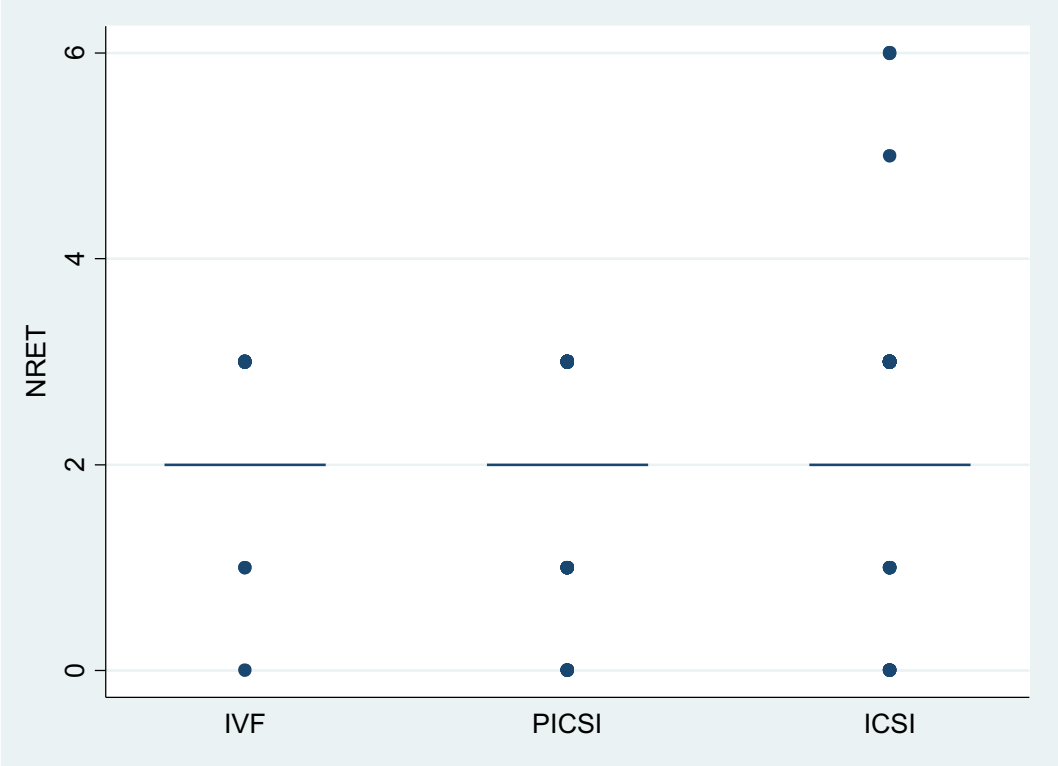
```
Defining indicator

generate pr_mis=0
replace pr_mis=1 if PR==3 | PR==31 | PR==32 | PR==33

. tabulate proc3 pr_mis, chi2 exact row


           |        pr_mis
     proc3 |         0          1 |     Total
-----------+----------------------+----------
       IVF |        40          1 |        41
           |     97.56       2.44 |    100.00
-----------+----------------------+----------
     PICSI® |       154          9 |       163
           |     94.48       5.52 |    100.00
-----------+----------------------+----------
      ICSI |       176          8 |       184
           |     95.65       4.35 |    100.00
-----------+----------------------+----------
     Total |       370         18 |       388
           |     95.36       4.64 |    100.00

          Pearson chi2(2) =   0.7707   Pr = 0.680
           Fisher's exact =                 0.779
```

o) Embryo Transfers

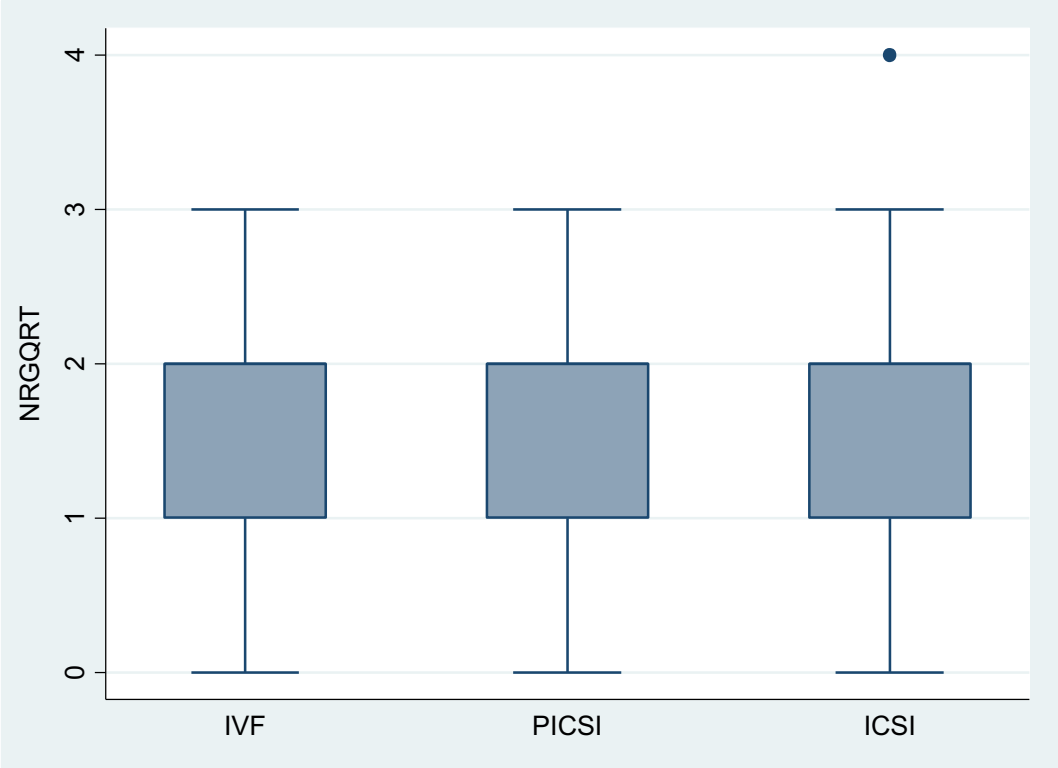
```
. tabstat NRET NRGQRT , statistics( mean sd count p25 median p75  ) by(proc3) varw
> idth(14) columns(statistics)
```

proc3	variable	mean	sd	N	p25	p50
IVF	NRET	2.073171	.5652541	41	2	2
	NRGQRT	1.65	.6998168	40	1	2
PICSI®	NRET	1.969325	.5602393	163	2	2
	NRGQRT	1.56051	.7623982	157	1	2
ICSI	NRET	2.13587	.7377534	184	2	2
	NRGQRT	1.662921	.801747	178	1	2
Total	NRET	2.059278	.6542183	388	2	2
	NRGQRT	1.618667	.7747163	375	1	2

proc3	variable	p75
IVF	NRET	2
	NRGQRT	2
PICSI®	NRET	2
	NRGQRT	2
ICSI	NRET	2
	NRGQRT	2
Total	NRET	2
	NRGQRT	2



- Boxplot: double embryo return is the standard practice in all procedure groups



- Identical embryo transfer profiles

NRET	proc3 IVF	PICSI®	ICSI	Total
0	1	6	6	13
	2.44	3.68	3.26	3.35
1	2	10	6	18
	4.88	6.13	3.26	4.64
2	31	130	137	298
	75.61	79.75	74.46	76.80
3	7	17	32	56
	17.07	10.43	17.39	14.43
5	0	0	1	1
	0.00	0.00	0.54	0.26
6	0	0	2	2
	0.00	0.00	1.09	0.52
Total	41	163	184	388
	100.00	100.00	100.00	100.00

Fisher's exact = 0.536

- Overall no difference in profiles p=.536

NRPQET	proc3 IVF	PICSI®	ICSI	Total
0	26	103	114	243
	65.00	65.61	64.04	64.80
1	10	34	37	81
	25.00	21.66	20.79	21.60
2	3	17	20	40
	7.50	10.83	11.24	10.67
3	1	3	6	10
	2.50	1.91	3.37	2.67
4	0	0	1	1
	0.00	0.00	0.56	0.27
Total	40	157	178	375
	100.00	100.00	100.00	100.00

Fisher's exact = 0.981

- profiles are very similar

ANOVA NRET

Number of obs = 388 R-squared = 0.0145
Root MSE = .651134 Adj R-squared = 0.0094

Source	Partial SS	df	MS	F	Prob>F
Model	2.4062235	2	1.2031117	2.84	0.0598
proc3	2.4062235	2	1.2031117	2.84	0.0598
Residual	163.23037	385	.423975		
Total	165.6366	387	.42800155		

ANOVA NRPQET

Number of obs = 375 R-squared = 0.0018
Root MSE = .81179 Adj R-squared = -0.0035

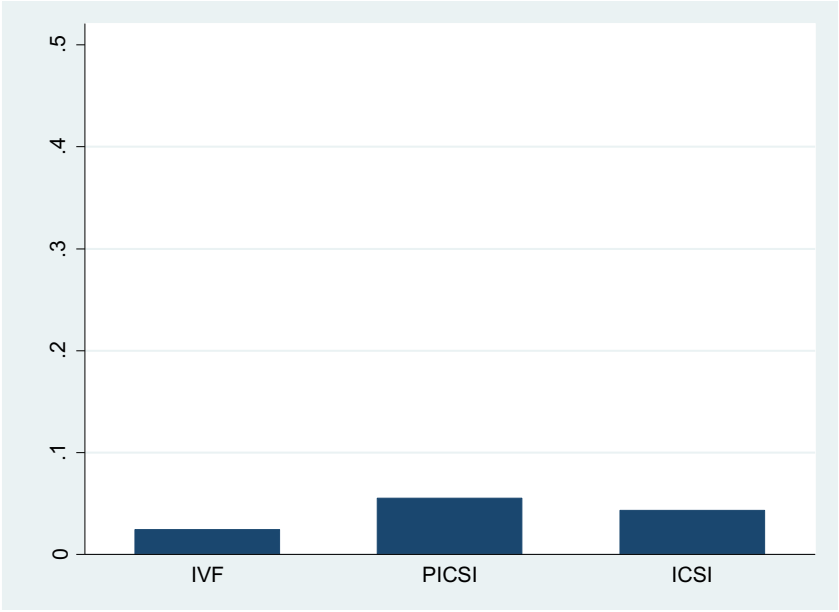
Source	Partial SS	df	MS	F	Prob>F
Model	.45112896	2	.22556448	0.34	0.7104
proc3	.45112896	2	.22556448	0.34	0.7104
Residual	245.14887	372	.65900234		
Total	245.6	374	.65668449		

Quantile regression model:

```
. xi: qreg NRET i.proc3  , quantile(50)
i.proc3           _Iproc3_0-2         (naturally coded; _Iproc3_0 omitted)
Iteration  1:  WLS sum of weighted deviations =  57.229663

Median regression                               Number of obs =        388
  Raw sum of deviations     55.5 (about 2)
  Min sum of deviations     55.5                Pseudo R2     =     0.0000

------------------------------------------------------------------------------
        NRET |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Iproc3_1 |          0  (omitted)
   _Iproc3_2 |          0  (omitted)
       _cons |          2          .        .       .            .           .
------------------------------------------------------------------------------
```

```
. xi: qreg NRGQRT i.proc3  , quantile(50)
i.proc3           _Iproc3_0-2         (naturally coded; _Iproc3_0 omitted)

Median regression                                 Number of obs =        375
  Raw sum of deviations     95.5 (about 2)
  Min sum of deviations     95.5                  Pseudo R2     =     0.0000

------------------------------------------------------------------------------
      NRGQRT |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Iproc3_1 |          0  (omitted)
   _Iproc3_2 |          0  (omitted)
       _cons |          2          .        .       .            .           .
------------------------------------------------------------------------------
```

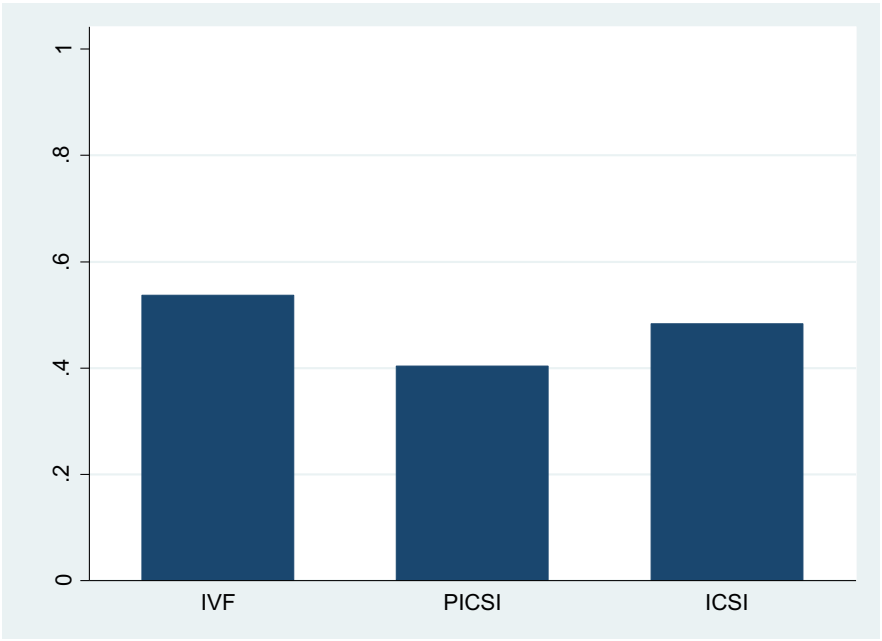
- no variability of any kind thus no significance tests for differences between the medians



p) Clinical Pregnancy

Nicole code the clinical pregnancy and I ran an analysis on that

```
. tabulate proc3 clin_pr, chi2 row

           |        clin_pr
     proc3 |         0          1 |     Total
-----------+----------------------+----------
       IVF |        19         22 |        41
           |     46.34      53.66 |    100.00
-----------+----------------------+----------
     PICSI® |        90         61 |       151
           |     59.60      40.40 |    100.00
-----------+----------------------+----------
      ICSI |        93         87 |       180
           |     51.67      48.33 |    100.00
-----------+----------------------+----------
     Total |       202        170 |       372
           |     54.30      45.70 |    100.00

          Pearson chi2(2) =   3.2605   Pr = 0.196


Overall no difference = 0.20
```



barchart looks similar to my own pr indicator

Some more modelling of risk difference (rd). Adjusted for incubator used, age of ova, procedure used (IVF,PICSI® ,ICSI),donor ova used, surrogate used and number of mii.

```
. xi: binreg clin_pr i.inc2 ageova i.proc3 i.donova i.surr mii, rd
i.inc2            _Iinc2_1-2          (naturally coded; _Iinc2_1 omitted)
i.proc3           _Iproc3_0-2         (naturally coded; _Iproc3_0 omitted)
i.donova          _Idonova_1-2        (_Idonova_1 for donova==0 omitted)
i.surr            _Isurr_0-1          (naturally coded; _Isurr_0 omitted)

Iteration 1:   deviance =  494.2373
Iteration 2:   deviance =  494.2327
Iteration 3:   deviance =  494.2327
Iteration 4:   deviance =  494.2327

Generalized linear models                         No. of obs      =        369
Optimization     : MQL Fisher scoring             Residual df     =        361
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  494.2326504                   (1/df) Deviance =   1.369066
Pearson          =  369.0002449                   (1/df) Pearson  =   1.022161

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = u                       [Identity]

                                                  BIC             =  -1639.565

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Risk Diff.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Iinc2_2 |   .0482779   .0733129     0.66   0.510    -.0954128    .1919686
      ageova |   .0085928   .0077341     1.11   0.267    -.0065657    .0237514
   _Iproc3_1 |   -.152674   .0871449    -1.75   0.080    -.3234748    .0181268
   _Iproc3_2 |  -.0766151   .0860183    -0.89   0.373    -.2452078    .0919777
  _Idonova_2 |   .1953642   .0808181     2.42   0.016     .0369636    .3537648
    _Isurr_1 |  -.0306542   .1606965    -0.19   0.849    -.3456136    .2843051
         mii |   .0077962   .0042126     1.85   0.064    -.0004604    .0160528
       _cons |   .1486018   .2748562     0.54   0.589    -.3901065    .6873101
------------------------------------------------------------------------------

Testing for overall procedure effect
. test _Iproc3_1 _Iproc3_2

 ( 1)  _Iproc3_1 = 0
 ( 2)  _Iproc3_2 = 0

           chi2(  2) =    3.80
```

```
         Prob > chi2 =    0.1497    no effect
```

q) Testing for overall procedure effect

```
. test _Iproc3_1= _Iproc3_2

 ( 1)  _Iproc3_1 - _Iproc3_2 = 0

           chi2(  1) =    1.93
         Prob > chi2 =    0.1644   no significant difference

Estimated difference in adjusted clinical pregnancy rate
. lincom _Iproc3_1- _Iproc3_2

 ( 1)  _Iproc3_1 - _Iproc3_2 = 0

------------------------------------------------------------------------------
     clin_pr |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |  -.0760589   .0546974    -1.39   0.164    -.1832638     .031146
------------------------------------------------------------------------------
```

PICSI® has 7.6% lower clinical pregnancy rate.

APPENDIX XVI Statistical Analysis Results (Prospective)

Clinical Pregnancy (Fetal Sac)

Unadjusted: Crude Analysis

FSAC	group: control	group: glue	Total
0	455	116	571
	67.61	58.88	65.63
1	172	52	224
	25.56	26.40	25.75
2	43	26	69
	6.39	13.20	7.93
3	3	3	6
	0.45	1.52	0.69
Total	673	197	870
	100.00	100.00	100.00

Pearson chi2(3) = 13.2800 Pr = 0.004

- Crude unadjusted comparison shows a significant difference in FSAC profile between groups, p=.004

```
. generate FSAC2=FSAC
(1 missing value generated)

. recode FSAC2 (1/max=1)
(FSAC2: 75 changes made)

Binary tabulation for FSAC
. tabulate FSAC2 group, chi2 column
```

Key
frequency
column percentage

FSAC2	group: control	group: glue	Total
0	455	116	571
	67.61	58.88	65.63
1	218	81	299
	32.39	41.12	34.37
Total	673	197	870
	100.00	100.00	100.00

Pearson chi2(1) = 5.1425 Pr = 0.023

- unadjusted comparison shows a significant difference in FSAC2 profile between groups, p=.004 (41% for glue versus 32% for control)

```
. xi:binreg FSAC2  group   , or

Generalized linear models                         No. of obs      =        870
Optimization     : MQL Fisher scoring             Residual df     =        868
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  1114.547297                   (1/df) Deviance =   1.284041
Pearson          =  869.9996593                   (1/df) Pearson  =   1.002304

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -4760.505
```

```
------------------------------------------------------------------------------
             |                 EIM
       FSAC2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       group |   1.457411   .2427861     2.26   0.024     1.051435    2.020139
       _cons |   .4791209   .0394656    -8.93   0.000     .4076908     .563066
------------------------------------------------------------------------------
```

- crude odds ratio = 1.46 (95% CI: 1.05 to 2.02) , p=.024

.

Number of Ova Per Patient

```
. tab NUMOVA , missing

     NUMOVA |      Freq.     Percent        Cum.
------------+-----------------------------------
          1 |         10        1.15        1.15
          2 |         36        4.13        5.28
          3 |         64        7.35       12.63
          4 |         53        6.08       18.71
          5 |         60        6.89       25.60
          6 |         57        6.54       32.15
          7 |         55        6.31       38.46
          8 |         41        4.71       43.17
          9 |         34        3.90       47.07
         10 |         38        4.36       51.44
         11 |         23        2.64       54.08
         12 |         31        3.56       57.63
         13 |         46        5.28       62.92
         14 |         32        3.67       66.59
         15 |         20        2.30       68.89
         16 |         21        2.41       71.30
         17 |         17        1.95       73.25
         18 |         16        1.84       75.09
         19 |         12        1.38       76.46
         20 |         10        1.15       77.61
         21 |         10        1.15       78.76
         22 |          3        0.34       79.10
         23 |          4        0.46       79.56
         24 |          3        0.34       79.91
         25 |          7        0.80       80.71
         26 |          4        0.46       81.17
         27 |          2        0.23       81.40
         28 |          1        0.11       81.52
         29 |          3        0.34       81.86
         31 |          3        0.34       82.20
         32 |          2        0.23       82.43
         33 |          1        0.11       82.55
         34 |          2        0.23       82.78
         35 |          1        0.11       82.89
         40 |          2        0.23       83.12
         48 |          1        0.11       83.24
         50 |          1        0.11       83.35
          . |        145       16.65      100.00
------------+-----------------------------------
      Total |        871      100.00
```

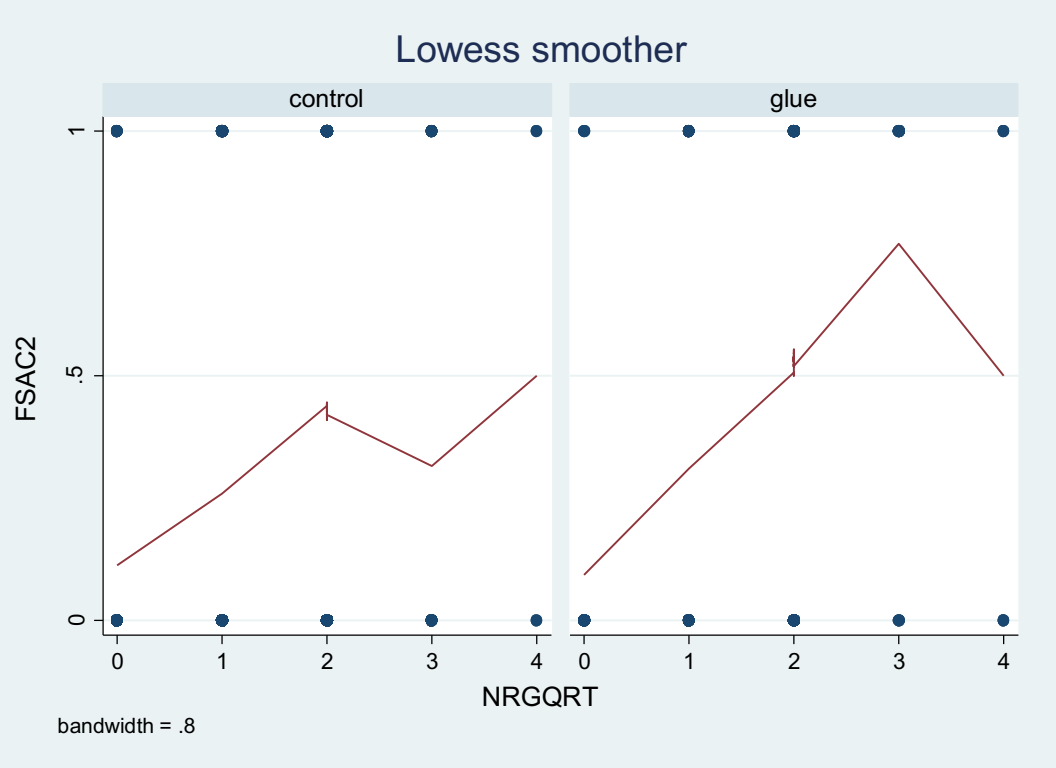
- 17% of records had missing ova data which is similar to the first analysis
- will do a model with and without this covariate

- logistic regression model- estimating odds ratios.
- number good quality embryos returned used as categorical outcome.

```
. xi:binreg FSAC2  group  DAYET i.DONOVA AGEOVA  i.NRGQRT NRET , or
i.DONOVA          _IDONOVA_0-1        (naturally coded; _IDONOVA_0 omitted)
i.NRGQRT          _INRGQRT_0-4        (naturally coded; _INRGQRT_0 omitted)

Generalized linear models                         No. of obs      =        868
```

```
Optimization     : MQL Fisher scoring              Residual df      =         858
                   (IRLS EIM)                     Scale parameter  =           1
Deviance         =  1009.657967                   (1/df) Deviance  =    1.176758
Pearson          =  886.1456204                   (1/df) Pearson   =    1.032804

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC              =   -4795.735
```

FSAC2	Odds Ratio	EIM Std. Err.	z	P>|z|	[95% Conf.	Interval]
group	1.340349	.2430039	1.62	0.106	.9394983	1.912229
DAYET	1.172206	.0785816	2.37	0.018	1.027878	1.336799
_IDONOVA_1	1.626416	.4046024	1.96	0.051	.998801	2.648406
AGEOVA	.991486	.0187284	-0.45	0.651	.9554503	1.028881
_INRGQRT_1	2.63016	.8231654	3.09	0.002	1.424231	4.857179
_INRGQRT_2	6.869349	2.033207	6.51	0.000	3.845695	12.27033
_INRGQRT_3	10.07205	4.340887	5.36	0.000	4.327767	23.44076
_INRGQRT_4	16.44466	12.32993	3.73	0.000	3.782758	71.48936
NRET	.7846531	.1121211	-1.70	0.090	.5929898	1.038265
_cons	.1161102	.0986886	-2.53	0.011	.0219476	.6142628




```
-> group = control
           |          FSAC2
      gnrt |         0          1 |     Total
-----------+----------------------+----------
         0 |        94         12 |       106
           |     88.68      11.32 |    100.00
-----------+----------------------+----------
         1 |       163         57 |       220
           |     74.09      25.91 |    100.00
-----------+----------------------+----------
         2 |       197        149 |       346   ( 2 or more)
           |     56.94      43.06 |    100.00
-----------+----------------------+----------
     Total |       454        218 |       672
           |     67.56      32.44 |    100.00
```

```
-------------------------------------------------------------------------------
-> group = glue
           |         FSAC2
      gnrt |         0          1 |     Total
-----------+----------------------+----------
         0 |        29          3 |        32
           |     90.63       9.38 |    100.00
-----------+----------------------+----------
         1 |        40         18 |        58
           |     68.97      31.03 |    100.00
-----------+----------------------+----------
         2 |        47         60 |       107  ( 2 or more)
           |     43.93      56.07 |    100.00
-----------+----------------------+----------
     Total |       116         81 |       197
           |     58.88      41.12 |    100.00
```

Same model as before but with grouped good number of embryos returned (gnrt)
The pooled variables provide stable estimates for this covariate

Model to report

```
. xi:binreg FSAC2  group  DAYET i.DONOVA AGEOVA  i.gnrt NRET , or
i.DONOVA          _IDONOVA_0-1        (naturally coded; _IDONOVA_0 omitted)
i.gnrt            _Ignrt_0-2          (naturally coded; _Ignrt_0 omitted)


Generalized linear models                         No. of obs      =        868
Optimization     : MQL Fisher scoring             Residual df     =        860
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  1011.974076                   (1/df) Deviance =   1.176714
Pearson          =  881.1282539                   (1/df) Pearson  =   1.024568

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -4806.951

------------------------------------------------------------------------------
             |                 EIM
       FSAC2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       group |   1.373411   .2472335     1.76   0.078     .9650996    1.954469
       DAYET |   1.165432   .0778451     2.29   0.022     1.022424    1.328444
  _IDONOVA_1 |   1.661143   .4114165     2.05   0.040     1.022326    2.699136
     AGEOVA |   .9949428    .018509    -0.27   0.785     .9593191    1.031889
    _Ignrt_1 |   2.725016   .8497077     3.21   0.001     1.478936    5.020981
    _Ignrt_2 |   7.142529   2.096908     6.70   0.000     4.017499    12.69838
        NRET |   .8622799   .1083063    -1.18   0.238     .6741142    1.102969
       _cons |    .086797    .071505    -2.97   0.003     .0172691     .436255
------------------------------------------------------------------------------
```

- adjusted odds ratio for glue is 1.37 (95%CI: .97 to 1.96) , p=.078. Thus the adjusted effect is not significant
- DAYET significant
- AGEOVA not significant
- Donor ova a significant factor
- NRQRT is strongly associated with FSAC2 with much higher odds for a positive outcome with 2 or more embryos returned (ngrt)
- NRET not significant
- interaction between group and good number returned is not significant, 0.4946 from results not shown.

With number of ova and more missing data

```
. xi:binreg FSAC2  group  DAYET i.DONOVA AGEOVA  i.gnrt NRET NUMOVA , or
i.DONOVA          _IDONOVA_0-1        (naturally coded; _IDONOVA_0 omitted)
i.gnrt            _Ignrt_0-2          (naturally coded; _Ignrt_0 omitted)
Generalized linear models                         No. of obs      =        726 (vs 868)
Optimization     : MQL Fisher scoring             Residual df     =        717
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  825.4726572                   (1/df) Deviance =   1.151287
Pearson          =  740.9955876                   (1/df) Pearson  =   1.033467
```

```
Variance function: V(u) = u*(1-u/1)              [Binomial]
Link function    : g(u) = ln(u/(1-u))            [Logit]

                                                 BIC             =  -3897.801
------------------------------------------------------------------------------
             |                 EIM
       FSAC2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       group |   1.408185   .2839809     1.70   0.090      .948424    2.090821
       DAYET |   1.193995   .1050304     2.02   0.044     1.004908    1.418662
  _IDONOVA_1 |   1.913229   .5675678     2.19   0.029     1.069681    3.421997
     AGEOVA |    .994941   .0217762    -0.23   0.817      .953163     1.03855
    _Ignrt_1 |   2.454154    .802143     2.75   0.006     1.293259    4.657126
    _Ignrt_2 |   6.844299   2.060154     6.39   0.000     3.794153    12.34648
        NRET |    .830324    .114445    -1.35   0.177     .6337602    1.087853
      NUMOVA |   1.017089   .0153375     1.12   0.261     .9874674    1.047598
       _cons |   .0798519   .0750233    -2.69   0.007     .0126635    .5035177
------------------------------------------------------------------------------
```

- Thus model without adjustment for number of ova not that different from a model without it but minimal missing data

Ongoing Clinical Pregnancy (Fetal Heart)

Unadjusted: Crude Analysis

```
           |         group
        FH |   control       glue |     Total
-----------+----------------------+----------
         0 |       463        120 |       583
           |     68.80      60.91 |     67.01
-----------+----------------------+----------
         1 |       155         49 |       204
           |     23.03      24.87 |     23.45
-----------+----------------------+----------
         2 |        53         27 |        80
           |      7.88      13.71 |      9.20
-----------+----------------------+----------
         3 |         2          1 |         3
           |      0.30       0.51 |      0.34
-----------+----------------------+----------
     Total |       673        197 |       870
           |    100.00     100.00 |    100.00

          Pearson chi2(3) =   7.4629   Pr = 0.059
```

```
           |         group
       FH2 |   control       glue |     Total
-----------+----------------------+----------
         0 |       463        120 |       583
           |     68.80      60.91 |     67.01
-----------+----------------------+----------
         1 |       210         77 |       287
           |     31.20      39.09 |     32.99
-----------+----------------------+----------
     Total |       673        197 |       870
           |    100.00     100.00 |    100.00

          Pearson chi2(1) =   4.2835   Pr = 0.038
```

```
. xi:binreg FH2  group , or

Generalized linear models                        No. of obs      =        870
Optimization     : MQL Fisher scoring            Residual df     =        868
                   (IRLS EIM)                    Scale parameter =          1
Deviance         =  1099.127015                  (1/df) Deviance =   1.266275
Pearson          =   869.999513                  (1/df) Pearson  =   1.002304

Variance function: V(u) = u*(1-u/1)              [Binomial]
Link function    : g(u) = ln(u/(1-u))            [Logit]

                                                 BIC             =  -4775.925
```

```
------------------------------------------------------------------------------
             |                 EIM
         FH2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       group |   1.414722   .2377494     2.06   0.039     1.017709    1.966613
       _cons |   .4535637   .0377351    -9.50   0.000     .3853195    .5338947
------------------------------------------------------------------------------
```

- crude odds ratio 1.41 (95% CI: 1.02 to 1.97) , p=.039 glue relative control

Adjusted model (without number of ova) – model to report

```
xi:binreg FH2  group  DAYET i.DONOVA AGEOVA  i.gnrt NRET  , or
i.DONOVA          _IDONOVA_0-1        (naturally coded; _IDONOVA_0 omitted)
i.gnrt            _Ignrt_0-2          (naturally coded; _Ignrt_0 omitted)

Generalized linear models                         No. of obs      =        868
Optimization     : MQL Fisher scoring             Residual df     =        860
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  1000.726327                   (1/df) Deviance =   1.163635
Pearson          =  882.3516975                   (1/df) Pearson  =    1.02599

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -4818.199

------------------------------------------------------------------------------
             |                 EIM
         FH2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       group |   1.338471   .2424758     1.61   0.108     .9384392    1.909025
       DAYET |   1.160714   .0781347     2.21   0.027     1.017246    1.324418
  _IDONOVA_1 |   1.528168   .3791739     1.71   0.087     .9396543    2.485272
     AGEOVA |   .9878271   .0184733    -0.65   0.513     .9522756    1.024706
    _Ignrt_1 |   2.524728   .7896279     2.96   0.003     1.367713     4.66052
    _Ignrt_2 |   6.643011   1.953776     6.44   0.000     3.732657    11.82257
        NRET |   .8157298   .1050124    -1.58   0.114     .6338222    1.049845
       _cons |   .1281487   .1058551    -2.49   0.013     .0253859    .6468974
------------------------------------------------------------------------------
```

With NUMBER OF OVA

```
xi:binreg FH2  group  DAYET i.DONOVA AGEOVA  i.gnrt NRET  NUMOVA , or
i.DONOVA          _IDONOVA_0-1        (naturally coded; _IDONOVA_0 omitted)
i.gnrt            _Ignrt_0-2          (naturally coded; _Ignrt_0 omitted)

Generalized linear models                         No. of obs      =        726
Optimization     : MQL Fisher scoring             Residual df     =        717
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  817.2487832                   (1/df) Deviance =   1.139817
Pearson          =  740.1390779                   (1/df) Pearson  =   1.032272

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -3906.025

------------------------------------------------------------------------------
             |                 EIM
         FH2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       group |   1.315619   .2672194     1.35   0.177     .8835683    1.958936
       DAYET |   1.225927   .1085062     2.30   0.021     1.030683    1.458156
  _IDONOVA_1 |   1.820598   .5391887     2.02   0.043     1.018877    3.253165
     AGEOVA |   .9860384   .0216619    -0.64   0.522     .9444829    1.029422
    _Ignrt_1 |   2.360404   .7731689     2.62   0.009     1.242135     4.48543
    _Ignrt_2 |   6.377573   1.922783     6.15   0.000      3.53204    11.51557
        NRET |   .7949667   .1122711    -1.62   0.104     .6027483    1.048484
      NUMOVA |   1.011153   .0152045     0.74   0.461      .981788    1.041397
       _cons |   .1147275   .1079861    -2.30   0.021     .0181336    .7258588
------------------------------------------------------------------------------
```

- estimates show some small changes, glue odds smaller, 1.32 compared to 1.34. Thus missing data is has no serious bias or effect.

Miscarriage Rates

Unadjusted: Crude Analysis

```
           |         group
      abrt |   control       glue |     Total
-----------+----------------------+----------
         0 |       637        184 |       821
           |     94.51      93.40 |     94.26
-----------+----------------------+----------
         1 |        37         13 |        50
           |      5.49       6.60 |      5.74
-----------+----------------------+----------
     Total |       674        197 |       871
           |    100.00     100.00 |    100.00

          Pearson chi2(1) =   0.3467   Pr = 0.556
```

```
. xi:binreg abrt  group   , or


Generalized linear models                         No. of obs      =        871
Optimization     : MQL Fisher scoring             Residual df     =        869
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  382.4989498                   (1/df) Deviance =   .4401599
Pearson          =  870.9674498                   (1/df) Pearson  =   1.002264

Variance function: V(u) = u*(1-u)                 [Bernoulli]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =   -5500.32

------------------------------------------------------------------------------
             |                 EIM
        abrt | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       group |   1.216363   .4051655     0.59   0.557     .6331864    2.336657
       _cons |   .0580848   .0098223   -16.83   0.000     .0416987      .08091
------------------------------------------------------------------------------
```

- odds for miscarriage not different between the glue and non-glue cycles , or=1.22 (95%CI: .63 to 2.34) , p=.557
- Thus no further adjustment was made

Subgroup Analysis for Descriptive Purposes

Age of Ova

```
           |         group
     ovage |   control       glue |     Total
-----------+----------------------+----------
         0 |       416        115 |       531
           |     61.72      58.38 |     60.96
-----------+----------------------+----------
         1 |       258         82 |       340   (36+)
           |     38.28      41.62 |     39.04
-----------+----------------------+----------
     Total |       674        197 |       871
           |    100.00     100.00 |    100.00

          Pearson chi2(1) =   0.7169   Pr = 0.397
```

- age distribution the same between the groups, p=.397

Embryos Transferred

```
           |         group
      NRET |   control       glue |     Total
```

```
----------+----------------------+----------
        1 |       108          45 |       153
          |     16.02       22.84 |     17.57
----------+----------------------+----------
        2 |       421         107 |       528
          |     62.46       54.31 |     60.62
----------+----------------------+----------
        3 |       123          31 |       154
          |     18.25       15.74 |     17.68
----------+----------------------+----------
        4 |        19          13 |        32
          |      2.82        6.60 |      3.67
----------+----------------------+----------
        5 |         3           0 |         3
          |      0.45        0.00 |      0.34
----------+----------------------+----------
        6 |         0           1 |         1
          |      0.00        0.51 |      0.11
----------+----------------------+----------
    Total |       674         197 |       871
          |    100.00      100.00 |    100.00

          Pearson chi2(5) =  16.4762   Pr = 0.006
```

```
          |         group
    nebet |   control       glue |     Total
----------+----------------------+----------
        1 |       108          45 |       153
          |     16.02       22.84 |     17.57
----------+----------------------+----------
        2 |       566         152 |       718  (2 or more embryos returned)
          |     83.98       77.16 |     82.43
----------+----------------------+----------
    Total |       674         197 |       871
          |    100.00      100.00 |    100.00

          Pearson chi2(1) =   4.8951   Pr = 0.027
```

- number of embryos returned is significantly different between the groups

Donor Ova

```
          |         group
   DON OVA |   control       glue |     Total
----------+----------------------+----------
        0 |       554         144 |       698
          |     82.20       73.10 |     80.14
----------+----------------------+----------
        1 |       120          53 |       173
          |     17.80       26.90 |     19.86
----------+----------------------+----------
    Total |       674         197 |       871
          |    100.00      100.00 |    100.00

          Pearson chi2(1) =   7.9299   Pr = 0.005
```

- number of donor ova used is more in the glue group 27% versus 674

Cycle Number

```
. tabulate CYCLE group, chi2 col

+--------------------+
| Key                |
|--------------------|
|     frequency      |
| column percentage  |
+--------------------+
```

CYCLE	group: control	glue	Total
0	1	0	1
	0.19	0.00	0.15
1	308	80	388
	57.46	53.33	56.56
2	126	33	159
	23.51	22.00	23.18
3	50	19	69
	9.33	12.67	10.06
4	23	8	31
	4.29	5.33	4.52
5	11	6	17
	2.05	4.00	2.48
6	11	3	14
	2.05	2.00	2.04
7	1	0	1
	0.19	0.00	0.15
9	2	0	2
	0.37	0.00	0.29
10	2	0	2
	0.37	0.00	0.29
11	0	1	1
	0.00	0.67	0.15
15	1	0	1
	0.19	0.00	0.15
Total	536	150	686
	100.00	100.00	100.00

Pearson chi2(11) = 9.3766 Pr = 0.587

cycle2	group: control	glue	Total
1	309	80	389
	57.65	53.33	56.71
2	227	70	297 (2nd or later cycle)
	42.35	46.67	43.29
Total	536	150	686
	100.00	100.00	100.00

Pearson chi2(1) = 0.8892 Pr = 0.346

- no difference in number of cycles between the groups