A controlled randomised study to compare the IUI biochemical pregnancy outcome between a routine swim-up and the Sep-D Kit semen preparation method

ROXANNE GENTIS

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Supervisor: Dr. Marie-Lena Windt De Beer

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the 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 2013

ABSTRACT

Male factor infertility is a general term that describes couples in which an inability to conceive is associated with a problem identified in the male partner. Intrauterine insemination (IUI) together with ovulation induction has been shown to be an effective treatment method for male factor infertility. Oocyte production by the ovaries is stimulated by the use of fertility drugs. A prepared sperm sample is then injected into the uterus through the vagina using an IUI catheter which brings the oocytes and spermatozoa into close proximity.

Semen preparation is an integral part of an IUI cycle. In a developing country, a simple inexpensive semen preparation method for IUI procedures, not necessitating a lot of equipment, is essential. An example of such a method, the Sep-D Kit (Surelife Sep-D Kit, Surelife Media Technologies Pty Ltd, Singapore) has been proposed as a possible preparation method. In a pilot study performed by the principal investigator (Roxanne Gentis), comparing the Sep-D Kit and standard swim-up preparation methods, it was found that the Sep-D Kit compared very well with the swim-up method regarding most pre- and post-preparation semen parameters. The Sep-D Kit method, however, still needed further testing to see whether or not pregnancy rates resulting from the method are comparable with that resulting from the standard swim-up method, as this ultimately is the required result of an IUI.

The primary aim of this study was to compare the Sep-D Kit method to the standard swim-up method with regards to biochemical pregnancy outcome, post-preparation

sperm count, motility, total motile count (TMC), morphology, DNA compaction and fragmentation (CMA_3 and TUNEL). The secondary aim was to evaluate which variables, male and female, affect biochemical pregnancy outcome.

The study took place at Drs Aevitas Fertility Clinic, Vincent Pallotti Hospital, Pinelands. The study was a prospective analytical study and was conducted from December 2010 until October 2012. A total of 473 IUI cycles were evaluated.

Results showed that the Sep-D Kit semen preparation method was non-inferior to the standard swim-up method with regards to biochemical pregnancy rates, post-preparation count and TMC. The swim-up method produced samples with a significantly higher post-preparation motility compared to the Sep-D Kit method, however both methods still managed to produce similar biochemical pregnancy rates (10.39% for the swim-up group versus 11.57% for Sep-D Kit group). For the total cohort of cycles analysed the only female parameter which significantly predicted biochemical pregnancy outcome in this study was age. Sperm motility (post-preparation) was the only male parameter that significantly affected biochemical pregnancy outcome.

The Sep-D Kit method is more cost effective and also time saving compared to the swim-up method. There is also no need for expensive laboratory equipment or a trained embryologist using the Sep-D Kit preparation method. The Sep-D Kit may therefore be used with confidence as a standard semen preparation method, and may be implemented in developing countries for use in routine IUI procedures.

OPSOMMING

Manlike faktor infertiliteit is 'n algemene term wat gebruik word om paartjies te beskryf wat 'n onvermoë toon om swanger te raak as gevolg van ‘n probleem wat geassosieer word met die man. Die kombinasie van intra-uteriene inseminasie (IUI) en ovulasie induksie kan doeltreffend gebruik word om manlike faktor infertiliteit te behandel. Vrugbaarheidsmiddels word gebruik om oösietproduksie in die die eierstokke te stimuleer en ‘n voorbereide spermmonster word dan transvaginaal in die baarmoeder ingespuit om sodoende die spermatozoa en oösiete na-aan mekaar te bring.

Semenvoorbereiding is 'n integrale deel van 'n IUI siklus en in 'n ontwikkelende land is 'n eenvoudige, goedkoop semenvoorbereidingsmetode – wat die gebruik van duur toerusting uitsluit – noodsaaklik. Die Sep-D Kit metode (Surelife Sep-D Kit, Surelife Media Technologies Pty Ltd, Singapore) is ‘n voorbeeld van so ‘n voorbereidingsmetode. 'n Loodsstudie, uitgevoer deur die hoofnavorser, (Roxanne Gentis), het gewys dat die Sep-D Kit en standaard opswem voorbereidingmetodes goed vergelyk ten opsigte van meeste semenparameters voor- en na voorbereiding. Dit is egter ook noodsaaklikheid vir verdere navorsing om vas te stel of swangerskapuitkoms na die gebruik van die twee semenvoorbereidingsmetodess vergelykbaar is, aangesien dit die uiteindelike, verlangde uitkoms van 'n IUI is.

Die primêre doel van hierdie studie was om die Sep-D Kit metode te vergelyk met die standaard opswemmetode met betrekking tot biochemiese swangerskapuitkoms asook spermtelling, motiliteit, totale motiele spermtelling (TMS), morfologie, DNA kompaksie

en fragmentering (CMA_3 en TUNEL) na spermvoorbereiding. Die sekondêre doel was om te evalueer watter veranderlikes, manlik en vroulik, die bichemiese swangerskapuitkoms beïnvloed.

Die studie is uitgevoer by die Drs Aevitas Fertiliteitskliniek, Vincent Pallotti Hospitaal, Pinelands. Die studie was prospektief analities en het gestrek vanaf Desember 2010 tot en met Oktober 2012. ‘n Totaal van 473 IUI siklusse is evalueer en ontleed.

Die resultate van die studie het getoon dat die Sep-D Kit semenvoorbereidingsmetode nie ondergeskik aan die opswemmetode was ten opsigte van biochemiese swangerskap, spermtelling en TMS na semenvoorbereiding nie, Spermmotiliteit was betekenisvol hoër vir die opswemmetode vergelykend met die Sep-D Kit, maar ten spite van die verskil was die biochemiese swangerskapsyfers in die twee groepe nie verskillend nie (10.39% in die opswem groep en 11.57% in Sep-D Kit groep). In die totale kohort siklusse wat ontleed is was dit net die ouderdom van die vrou wat ‘n betekenisvolle effek op biochemiese swangerskapuitkoms gehad het. Die enigste manlike faktor wat ‘n betekenisvolle effek op biochemiese swangerskapuitkoms gehad het was die motiliteit na semenvoorbereiding.

Die Sep-D Kit metode is meer koste-effektief en tydbesparend as die standard opswemmetode. Die uitvoer van die Sep-D Kit metode vereis ook ook geen duur apparaat of ‘n opgeleide embrioloog nie. Die Sep-D Kit metode kan dus met vertroue

gebruik word as 'n standaard semenvoorbereidingsmetode en kan in ontwikkelende lande vir gebruik tydens roetine IUI prosedures geïmplementeer word.

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2. Gentis RK, Siebert I, Kruger TF, De Beer-Windt ML. Implementation of an office-based semen preparation method (SEP-D Kit) for intra-uterine insemination (IUI): A controlled randomised study to compare the IUI pregnancy outcome between a routine (swim-up) and SEP-D Kit semen preparation method. *SAJOG* 2011;18(1):2-3

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LIST OF ABBREVIATIONS

IUI – Intrauterine insemination

ART – Assisted reproductive techniques

ICSI – Intracytoplasmic sperm injection

HCG – Human chorionic gonadotropin

βhCG – Beta human chorionic gonadotropin

HIV - Human Immunodeficiency Virus

DNA – Deoxyribonucleic acid

TMC – Total motile count

TVC – Total vital count

CMA_3 – Chromomycin A_3

ROS – Reactive oxygen species

TUNEL - Terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling

PBS – Phosphate buffered saline

CO_2 - Carbon dioxide

WHO – World Health Organisation

µm – Micrometres

mm – Millimetres

cm - Centimetres

ml – Millilitres

CHAPTER 1

1. INTRODUCTION

1.1 INTRAUTERINE INSEMINATION

Infertility is defined as the inability to conceive after twelve months of unprotected intercourse, and affects up to 15% of all couples of reproductive age (Huang *et al.,* 2012). The problem can be due to either a female factor (30%) or a male factor (30%) and in the rest of cases a combination of the two. The risk of infertility can also be increased and affected by overall poor health and lifestyle, including the misuse of drugs and alcohol, smoking, medicines as well as environmental toxins (Windt, Hoogendijk and Tinney, 2007). Male factor infertility is a general term that describes couples in which an inability to conceive is associated with a problem identified in the male partner. Many couples with male infertility are not absolutely infertile (nearly zero chance of becoming spontaneously pregnant) but are subfertile (reduced fertility with prolonged time of unwanted non-conception). For these couples, simple methods of assisted reproduction can help. In subfertility, generally less invasive and less expensive methods are tried first before proceeding to more complicated and expensive treatments (Nuojua-Huttunen *et al*., 1999). Intrauterine insemination (IUI), also known as artificial insemination, has been shown to be effective in the treatment of male factor subfertility (Kucuk *et al*., 2008). IUI is a simple, inexpensive, effective form of therapy (Nuojua-Huttunen *et al*., 1999). The first paper entitled IUI was published in 1962 (Cohen, 1962) and since then IUI has evolved through sperm preparation and ovulation induction. Ovulation induction drugs such as Clomid (clomiphene citrate) are used to stimulate oocyte production to increase the chances of success by increasing the

gamete density at the site of fertilization (Ombelet., 2004). A prepared, washed sperm sample is injected into the uterus at the time of ovulation, through the vagina, by means of a catheter, which brings the sperm and oocytes into close proximity (Figure 1.1).

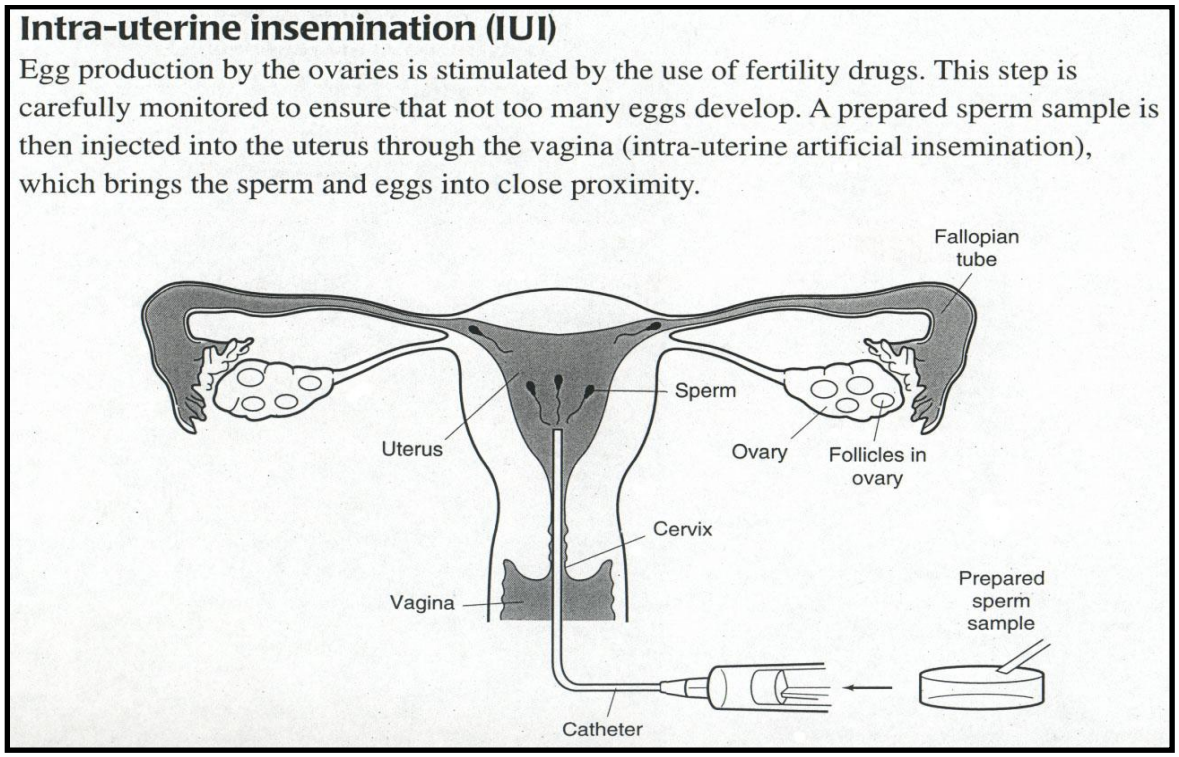


Figure 1.1 Schematic representation of the process of Intrauterine Insemination (IUI)
From: Merck Serono Patient Information Brochure

Female patients are stimulated from day 4 to 8 with either 50mg or 100mg Clomid. An ultrasound is performed on day 11 of the patients' cycle and if follicles greater than 18mm are observed the patient received HCG (human chorionic gonadotropin), more commonly known as the trigger shot, in order to stimulate ovulation. Insemination is then performed 36 hours post HCG (Abdelkader and Yeh, 2009).

1.2 FEMALE FACTORS AND DIAGNOSIS

According to Montanaro Gauci *et al.* (2001) there is a linear association between the number of follicles and the pregnancy risk ratio (chance). Nuojua-Huttunen *et al.* (1999) agrees with Montanaro Gauci *et al.* (2001) that the number of follicles present is a good predictor of IUI outcome. They also state that pregnancy rates were remarkably higher when three pre-ovulatory follicles were present. A simple explanation for the increased pregnancy rates is that multifollicular development results in an increased number of fertilizable oocytes and a better quality endometrium, thereby improving fertilization and implantation rates. On the other hand, the risk of multiple pregnancies increase with an increasing follicle number, and therefore careful monitoring remains essential (Ombelet., 2004).

Palatnik *et al.* (2012) found that there is an optimal size for the leading follicle that maximizes the probability of pregnancy. Higher pregnancy rates were achieved with the leading follicle being in the range of 23 to 28mm. Within that range, pregnancy rates were higher when the larger follicles were accompanied by a thicker endometrium. The relationship between the leading follicular size and the probability of pregnancy was found to be closely related to the endometrial thickness. This reflects the co-ordination between follicular growth and the endometrial lining. During the menstrual cycle, the endometrium undergoes cyclic changes. Larger follicles would be expected to produce higher levels of estradiol that would in turn stimulate the endometrial lining to produce a thicker lining, while smaller follicles would produce lower levels of estradiol and thus produce a thinner endometrial lining. When this co-ordination is disrupted, lower

pregnancy rates will result. The endometrial thickness is therefore a predictive factor of achieving pregnancy (Palatnik *et al.* 2012).

Most pregnancies occur within the first three attempts and the chances of success per month drop considerably after the fourth attempt. IUI treatment is therefore recommended for a maximum of three to four tries (Tomlinson *et al*., 1996; Shulman *et al*., 1998; Nuojua-Huttunen *et al*., 1999; Dickey *et al*., 2002). The timing of the IUI is very important because the oocytes are only fertilizable for 12-24 hours after ovulation. Insemination should therefore occur at or slightly before the time of ovulation (Abdelkader and Yeh, 2009). Semen is occasionally inserted twice within a treatment cycle. This double intrauterine insemination has been theorized to increase pregnancy rates by decreasing the risk of missing the fertile window during ovulation. However, a randomized trial of insemination after ovarian hyperstimulation found no difference in live birth rate between single and double intrauterine insemination (Bagis *et al*., 2010). One factor that did play a role is female age. It was found that there is a linear (negative) association between female age and pregnancy (Montanaro Gauci *et al*., 2001). The age-related decline in female fecundity has been suggested as a result of a reduced uterine receptivity and/or decreased oocyte quality (Nuojua-Huttunen *et al.,* 1999). It has been noted that IUI is a poor treatment option for women over the age of 40 years (Campana *et al*., 1996; Nuojua-Huttunen *et al*., 1999; Zadehmodarres *et al*., 2009). Various studies prove that the duration of infertility is a prognostic factor in predicting pregnancy rates (Tomlinson *et al.,* 1996; Nuojua-Huttunen *et al.,* 1999; Zadehmodarres *et al.,* 2009), however not all studies agree with this. Although there is not any precise limits of the duration of infertility, after which IUI success has been

shown to decrease, IUI cannot be recommended to patients with a long-standing duration of infertility (Zadehmodarres *et al.,* 2009).

The IUI procedure can also be an effective form of treatment for some causes of female infertility such as cervical factor infertility (including sperm antibodies), mild endometriosis, anovulation and unexplained infertility (Campana *et al*., 1996; Tomlinson *et al*., 1996; Zadehmodarres *et al*., 2009; Merviel *et al*., 2010). The best results were obtained in cervical indications, followed by anovulation, male-factor infertility, unexplained infertility and lastly endometriosis (Merviel *et al.,* 2010). IUI is successful because it bypasses the cervix, the ovulation cycle is accurately observed and controlled, semen is washed to increase the total number of motile sperm present for insemination and ovulation drugs stimulate oocyte production increasing the number of possible fertilizable oocytes. The negative impact of endometriosis on IUI success has been widely reported (Nuojua-Huttunen *et al.,* 1999; Dickey *et al.,* 2002; Merviel *et al.,* 2010) and it has been suggested that cytokines and growth factors secreted by the endometrial tissue could interfere with ovulation, fertilization, implantation and embryonic development (Merviel *et al.,* 2010).

1.3 SEMEN PREPARATION

Semen preparation (sperm washing) is an integral part of an IUI cycle. Semen processing methods are designed to enhance sperm function and increase the chances of conception by positively affecting motility and morphology; however, it negatively affects the total sperm count (Henkel and Schill, 2003; Kucuk *et al*., 2008). Only washed and prepared sperm may be used for IUI because neat semen may cause severe uterine contractions, pain and cramps due to prostaglandins in the semen. The aim of washing and preparation of sperm are to separate sperm from seminal plasma, remove bacteria, leukocytes and other chemicals and debris that may cause infection and irritation. It is also performed in order to improve sperm capacitation (the ability of sperm to penetrate and fertilize an oocyte) and to decrease the risk of transferring HIV in HIV positive patients (Henkel and Schill, 2003). There are four basic approaches to sperm preparation: 1) Simple dilution and washing, also known simply as swim-up 2) Sperm migration 3) Density gradient centrifugation 4) Adherence methods e.g. glass wool, glass beads, and Sephadex columns (Henkel and Schill, 2003). The sperm preparation method is determined by the quality of the sample produced for IUI; therefore macroscopic and microscopic analysis of the sample is first required. Factors that influence the decision of which sperm preparation technique should be used are: the percentage of motile sperm, the rate of forward progression, concentration and the number of other cells present in the sample (Mortimer, 2000; Henkel and Schill, 2003). The sperm sample used in IUI is mostly prepared by either the wash and swim-up method or the gradient centrifugation method. In both methods, seminal plasma is removed and motile, fast-swimming spermatozoa are isolated. For the wash and swim-

up method, a semen sample with good motility, concentration and forward progression is required. Samples with decreased motility, count and forward progression as well as those with high viscosity, cells and debris are best prepared with the gradient centrifugation method (Windt, Hoogendijk and Tinney, 2007). Different methods of sperm washing can result in apparent differences of sperm recovery rates, nevertheless, no one method offers superior cycle fecundity to another (Dodson *et al.,* 1998). This can be explained by the fact that almost all methods of semen washing surpass the low threshold number of $1x10^6$ motile sperm needed for conception after an IUI (Ombelet *et al.,* 2003).

1.3.1 Sep-D Kit METHOD

In a developing country, a simple inexpensive semen preparation method for IUI procedures, not necessitating a lot of equipment is essential. An example of such a method, the Sep-D Kit (Surelife Sep-D Kit, Surelife Media Technologies Pty Ltd, Singapore) has been proposed as a possible preparation method. The Kit has 5 devices containing HEPES based sperm wash medium used for separating motile spermatozoa from semen samples for IUI (figure 1.2). This Kit has overcome the need for any laboratory equipment, including a Laminar Flow cabinet, CO_2 incubator, centrifuge and many tubes and pipettes. This method is suitable for the processing of all semen samples except for those samples with less than 2million/ml spermatozoa. Sep-D is a simple device, easy to use and the insemination catheter can be connected directly to the device for insemination. The culture medium contains amino acids and special nutrients to separate the most number of acrosome reacted and viable sperm with

normal DNA. The processing of semen using Sep-D does not involve centrifugation and hence there is no risk of any trauma to the sperm. The method is quick and avoids any unnecessary waiting. Overall the Sep-D Kit is cheaper than the standard swim-up method (R242.10 and R334.03 respectively per IUI insemination), it is more time efficient (2 hours needed to perform a swim-up whereas only 1 hour necessary for a Sep-D Kit), and the method is easier to perform. This method however, needs to be comparable in outcome to an already successful established method, namely the swim-up method.

Figure 1.2 Photograph of the Sep-D Kit device (syringe filled with medium)

Photo by Nicole Lans

In a pilot study performed by the principal investigator (Roxanne Gentis) comparing these two methods (n=29) regarding certain parameters pre- and post-preparation,

including concentration, motility, vitality, morphology, DNA integrity and also Total Motile Count (TMC) and Total Vital Count (TVC), it was found that the Sep-D Kit compared very well with the standard swim-up method. The TMC is an indication of the total number of motile spermatozoa in the sample available for insemination and this is significant when comparing the two samples. The Sep-D Kit method proved overall to have significantly more motile spermatozoa in the sample than the swim-up method (Figure 1.3). The TVC gives us an indication of the total number of vital (live) spermatozoa in the sample available for insemination and this is significant when comparing the two samples. The Sep-D Kit method proved overall to have significantly more live spermatozoa in the sample than the swim-up method (Figure 1.4).

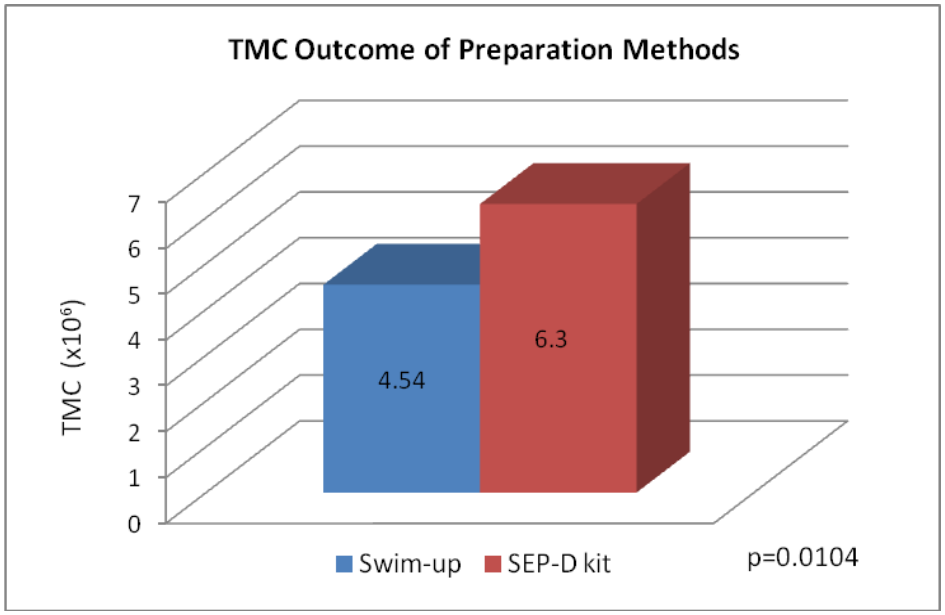


Figure 1.3 Histogram showing the significant difference in total motile count (TMC) after preparation with the swim-up and Sep-D Kit methods

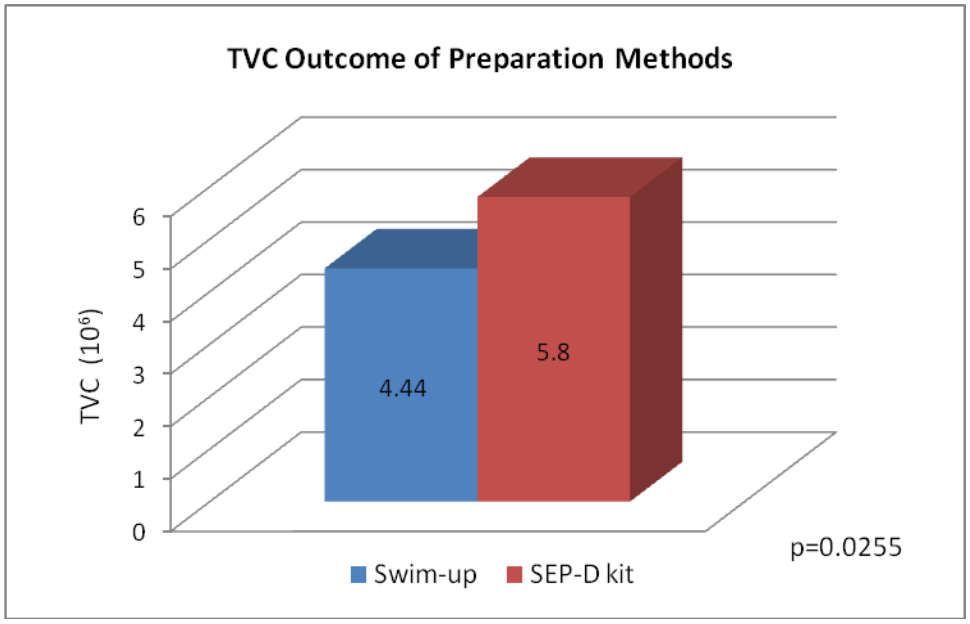


Figure 1.4 Histogram showing the significant difference in total vital count (TVC) after preparation with the swim-up and Sep-D Kit methods

In the study the Sep-D Kit method had a higher TMC and TVC than the swim-up method (figure 1.3 and figure 1.4); the Sep-D Kit is therefore comparable to the conventional swim-up method. The Sep-D Kit method may even be the better method as it is simple, fast and effective and also showed no difference in sperm DNA maturity (CMA_3).

Since in this pilot study the two methods compared favourable, the Sep-D Kit was rendered acceptable to be used for routine IUI procedures, however the method still needed further testing to see whether or not its pregnancy rates are comparable, as this ultimately is the required result of IUI.

1.4 MALE FACTORS

1.4.1. Concentration

The parameters studied in the pilot study are of importance since the total number of spermatozoa per ejaculate and the sperm concentration are related to both time to pregnancy and pregnancy rates and are predictors of conception (WHO, 1999). The number of spermatozoa in the ejaculate is calculated from the concentration of spermatozoa, which is measured during semen evaluation. For normal ejaculates, when the male tract is unobstructed and the abstinence time short, the total number of spermatozoa in the ejaculate is correlated with testicular volume (WHO, 1987) and thus is a measure of the capability of the testes to produce spermatozoa (MacLeod and Wang, 1979) and the patency of the male tract. The concentration of spermatozoa in the semen, while related to fertilization and pregnancy rates, is influenced by the volume of the secretions from the seminal vesicles and prostate and is not a specific measure of testicular function (WHO, 1999). Some articles state that the threshold value for sperm concentration for IUI should be greater than $1x10^6$ or the outcome will be adversely affected (Campana *et al*., 1996), while others state that 5 million total motile sperm before preparation represent threshold levels (Dickey *et al*., 2002). There are many conflicting articles however it has been shown that the number of inseminated sperm significantly affects the pregnancy rate.

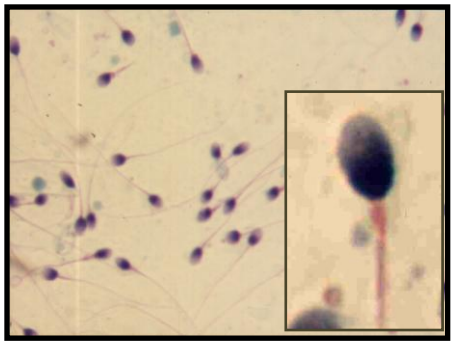
1.4.2. Motility

In determining quantitative motility one distinguishes the percentage of motile spermatozoa from the percentage of immotile spermatozoa. The estimation of the percent motile is made to the nearest 10 percent (Menkveld and Coetzee, 1995). The number of motile sperm inseminated is the contributing factor with the greatest impact on the chance of IUI pregnancy (van der Westerlaken *et al*., 1998). Also proven by Shulman *et al.* (1998) the degree of motility of inseminated sperm is known to be the major predictive factor for the success rate in IUI treatment. Therefore the extent of progressive sperm motility is related to pregnancy rates (Zinaman *et al*., 2000; Larsen *et al.*, 2000). During sperm preparation, improvement in sperm motility and forward progression is attained, as the sperm with the greatest motility are selected by the swim-up procedure. Due to the fact that there is a positive correlation between sperm motility and morphology, the latter can also be improved during semen preparation (Mortimer *et al.,* 1982).

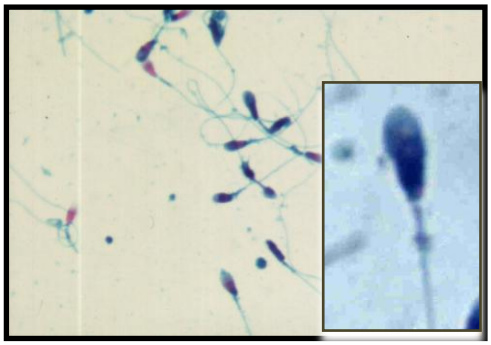
1.4.3 Morphology

To evaluate sperm morphology, semen smears are made and stained by different staining techniques. The most common technique being the Diff Quik staining technique (Appendix IV) as used at Vincent Pallotti Hospital and Tygerberg Hospital. Spermatozoa consist of a head, neck, middle piece (midpiece), principal piece and end piece. For a spermatozoon to be considered morphological normal, both its head and tail must be normal (Figure 1.5). All borderline forms should be considered abnormal. Men whose spermatozoa all display one of these defects are usually subfertile (WHO, 1999). The

criteria for a normal spermatozoon are as follows: 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 more than two small vacuoles, which should not occupy more than 20% of the sperm head. The post-acrosomal region should not contain any vacuoles. The midpiece should be slender, regular and about the same length as the sperm head. The major axis of the midpiece 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 and Menkveld, 2001). The principal piece should have a uniform calibre along its length, be thinner than the midpiece 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 (Figure 1.6).



Normal spermatozoa



Abnormal spermatozoa

Figure1.5 Photographs showing normal and abnormal sperm morphology

Photographs kindly provided by Dr. ML Windt De Beer

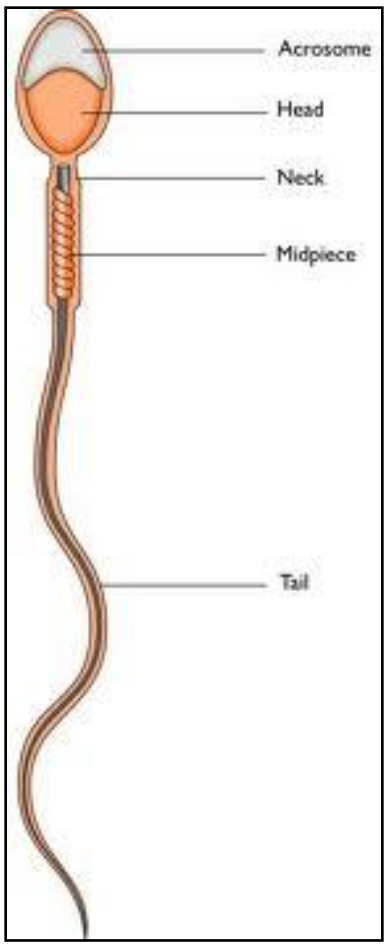


Figure 1.6 Schematic representation of a normal human sperm cell

http://www.turkey-ivf.com/ivf/normal_spermatozoa.html

The following head aberrations can be observed: head shape and/or size defects, including large, small, tapering, pyriform, amorphous, vacuolated, double heads, or any combination of these (WHO, 1999).

Neck and midpiece aberrations that can be observed are: complete absence, non-inserted, bent midpiece, or any combination of these (WHO, 1999).

Tail aberrations observed are: short, multiple, hairpin, broken, coiling, or any combination of these (WHO, 1999).

The morphology is assessed after using a staining procedure (Diff Quick), and the morphologic rating should include the counting of apparently normal spermatozoa. At the Fertility Clinics Tygerberg and Vincent Pallotti Hospitals, this is considered one of the most significant aspects of semen evaluation because it gives excellent information regarding fertility. This parameter is expressed as the percentage of normal forms or normal morphology (Kruger, 2007). When the sperm morphology is between 0% and 4% normal forms, the sample is considered a possibly infertile sample and known more commonly as the poor-pattern (p-pattern) morphology. When the sperm morphology is between 5% and 14% normal forms, the sample is considered a subfertile sample and known more commonly as the good-pattern (g-pattern) morphology. Finally when the sperm morphology is greater than 15% normal forms, the sample is considered a fertile sample and known more commonly as the normal-pattern (n-pattern) morphology. According to Montanaro Gauci *et al.* (2001) the percentage motility and percentage normal morphology (by strict criteria) of sperm in the fresh ejaculate are the male factors that significantly and independently predict the pregnancy outcome. Various other articles agree with Montanaro Gauci that the percentage normal morphology is a predictor of pregnancy outcome (Merviel *et al*., 2010; Ombelet *et al*., 2003; Dickey *et al*., 1999). Ombelet *et al.* (2003) states that the IUI success rate is impaired when a sample with less than 5% normal sperm morphology is inseminated.

1.4.4 DNA Integrity

The genetic status (DNA integrity) of the sperm cell is also very important in the testing of male fertility as it contributes to one half of the genomic material to offspring. Some assisted reproductive procedures (ART) such as intracytoplasmic sperm injection (ICSI) bypass natural selection mechanisms, which increases the chance that sperm with abnormal genomic material will fertilise an oocyte. Sperm DNA is organised in a unique pattern that keeps the chromatin in the nucleus compact and stable (Agarwal and Allamaneni, 2004). DNA damage may occur by at least three mechanisms: (i) defective chromatin condensation during spermiogenesis; (ii) initiation of apoptosis during spermatogenesis; (iii) by oxidative stress mainly resulting from reactive oxygen species (ROS) produced (Duran *et al*., 2002).

(i) Defective Chromatin Packaging

Immature sperm have high levels of DNA damage and ROS production, and are likely to have alterations in protamination and chromatin packaging (Sharma *et al*., 2004). In the presence of significant DNA damage, compact packaging via cross-linking of protamines by disulphide bonds becomes impossible (Filatov *et al.,* 1999).

(ii) Apoptosis

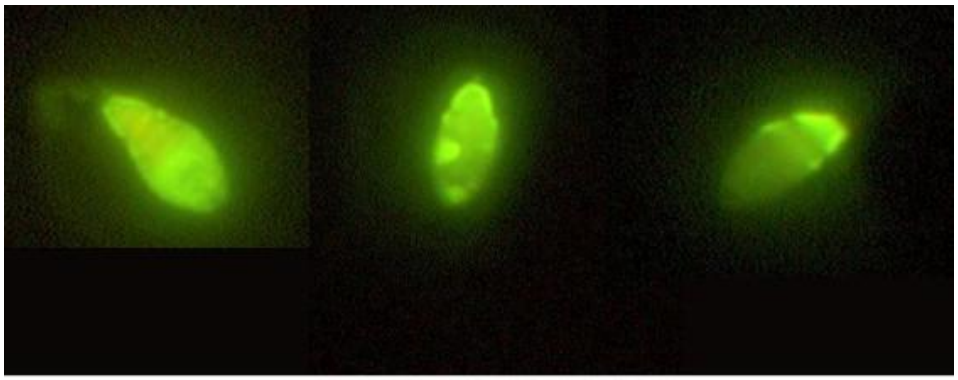
Apoptosis is programmed cell death and therefore controls the overproduction of sperm, so that the sperm does not surpass the supportive capacity of the Sertoli cells. Apoptosis occurs in the testis during spermatogenesis and generates numerous DNA strand breaks. Apoptosis may not always operate efficiently and the subsequent

ejaculated sperm are representative of cells in the process of apoptosis (Sharma *et al*., 2004).

(iii) Oxidative Stress and ROS

Oxidative stress is caused by an imbalance between the production of ROS by leukocytes or sperm and the antioxidant capacity of semen. ROS may lead to chromatin cross-linking and DNA strand breaks (Agarwal and Allamaneni, 2004).

Several tests can be used to study sperm DNA abnormalities. One of these tests is the Chromomycin A3 (CMA_3) test, which measures the chromatin packaging (maturity) in the sperm head and therefore identifies sperm chromatin packaging defects. The CMA_3 test therefore measures DNA damage after denaturation (Sakkas and Alvarez, 2010). Previous studies (Nijs *et al.,* 2009; Tavalaee *et al*., 2009) have found a strong correlation between CMA_3 results and sperm morphology. Another test used to identify the integrity of sperm DNA is the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) test. In the TUNEL test, terminal deoxynucleotidyl transferase incorporates dUTP biotinlyated deoyuridine to 3'-OH at single- and double-strand DNA breaks to create a fluorescent signal (Figure 1.7). By measuring the actual DNA strand breaks, the TUNEL test measures the DNA damage directly (Sakkas and Alvarez, 2010).



Note: TUNEL = TdT-mediated dUTP Nick-End Labeling.

Figure 1.7 Abnormal patterns of DNA fragmentation as seen under a fluorescent microscope subsequent to the TUNEL assay
Barroso *et al*., 2009.

Duran *et al.* (2002) studied the degree of sperm DNA fragmentation using TUNEL in predicting the success of IUI outcome. The article reported that the degree of DNA fragmentation after sperm preparation was significantly lower in the samples that produced pregnancies. The article also stated that no woman inseminated with a sample having greater than 12% of sperm with fragmented DNA achieved a pregnancy (Duran *et al*., 2002). Mahfouz *et al.* (2010) conducted a study in which sperm motility, DNA fragmentation (using the TUNEL test), and the medical history of infertile men with high seminal ROS was examined. This study reported that infertile men with high seminal ROS levels also have a high incidence of sperm DNA fragmentation, and that an increase of seminal ROS by 25% may be associated with a 10% increase in sperm DNA fragmentation. The sperm motility was found to be affected by seminal ROS and sperm DNA fragmentation, therefore the percentage of total motile sperm is negatively related to seminal ROS as well as sperm DNA fragmentation. Techniques such as the TUNEL assay and the sperm chromatin structure assay both show increased levels of

DNA abnormalities in spermatozoa from men who have poor semen parameters. The main reproductive parameter affected by an increased presence of DNA abnormalities in ejaculated spermatozoa is pregnancy rates (Spano *et al*., 2005).

1.5 AIMS AND OBJECTIVES

1.5.1 Primary objectives

To compare the Sep-D Kit method with the standard swim-up method with regards to:

- Biochemical Pregnancy outcome (IUI)
- Post preparation count, motility, morphology and Total Motile Count (TMC)

1.5.2 Secondary objectives

- To compare the Sep-D Kit method with the standard swim-up method with regards to DNA integrity (fragmentation and compaction)
- The role of the female diagnosis (age, endometrium thickness and number of follicles) and male factors on biochemical pregnancy rates

1.6 HYPOTHESIS

We hypothesize that:

a) the Sep-D Kit method will give similar results compared to the standard swim-up method with regards to IUI biochemical pregnancy outcome;

b) the Sep-D Kit method can replace the swim-up method in cases where an office based ART programme needs to be followed; since the Sep-D Kit method will give similar results with regards to post preparation count, motility, morphology and TMC

CHAPTER 2

2. MATERIALS AND METHODOLOGY

This study was a prospective analytical study and took place from December 2010 until October 2012. All patients (of any age) undergoing an IUI cycle and that fitted into the inclusion criteria was included in the study population. A total of 473 IUI cycles were evaluated, with 53 patients having one or more repeat cycles. A subgroup of 202 IUI cycles (of the 473 cycles) were evaluated for morphology and DNA integrity results. At the Drs Aevitas Fertility Clinic various medical scientists perform the semen washing technique, thus the principle investigator was only able to capture complete data sets for 202 IUI cycles.

All IUI patients included:

- Inclusion criteria:

  > 10 x 10^{6}/ml sperm

  > 40% motility

  ≥ 1.2 ml semen

- Using a randomised table (Appendix II), IUI cycles were assigned a method of preparation as they were booked
  - Sep-D Kit (n=242)
  - Swim-up (n=231)

- Noted before and after semen preparation:
    - Motility (%)
    - Count (millions/ml)
    - Total Motile Count (millions)
    - Sperm Morphology (%)
    - DNA maturity and integrity (CMA_3 and TUNEL)

- Analyzed results also according to the morphology groups:
    - 0-4% (p-pattern morphology)
    - ≥ 5% (g-pattern morphology)

- Exclusion Criteria:
    - ++(+) round cells
    - Viscosity >10cm
    - HIV positive samples

- Female factors included:
    - Age
    - Cycle number
    - Diagnosis
    - Number of follicles
    - Endometrium thickness
    - Biochemical pregnancy outcome

After receiving consent from patients; all female information (age, cycle number, diagnosis, number of follicles and endometrium thickness) was recorded on the designed IUI information form (Appendix I).

When IUI was booked, random selection of sperm washing technique to be used was determined by means of the randomised table (Appendix II).

On the day of the IUI, the male partner produced a semen sample for the selected semen preparation method.

2.1 Pre-preparation analysis:

After complete liquefaction at room temperature, the volume and viscosity of semen was determined according to World Health Organization criteria (WHO, 1999).

A wet prep was made by placing a 10µl drop of semen on a clean glass slide and covered with a 22mm x 22mm cover slip. From this, sperm motility, forward progression and estimated concentration, as well as the number of cells present were determined (Appendix III).

Two smears of the semen sample were made by applying a drop of semen, the size of the drop depending on sperm concentration, to the end of the slide. A second slide was used to pull the drop of semen along the surface of the slide (see figure 2.1 below). Slides were allowed to air dry at room temperature.

Figure 2.1 Schematic representation indicating the correct method to make a semen smear
(WHO, 1999)

One smear was used to conduct the Diff Quik staining technique and ascertain sperm morphology by following the Tygerberg Strict Criteria method (Appendix IV). The second smear was used to evaluate the chromatin packaging quality of the spermatozoa by conducting CMA_3 staining (Appendix V).

50µl sperm suspension with 150µl PBS was centrifuged for 10 minutes at 300xg. The supernatant was discarded and the pellet resuspended in 150µl PBS. This process was repeated. 50µl of sperm suspension was pipetted onto a starfrost slide and a smear was made. Slides were allowed to air dry at room temperature. The washed, air dried smear was used to conduct the TUNEL assay (Appendix VI) in order to evaluate DNA fragmentation which is a hallmark of apoptosis.

2.2 Semen preparation

2.2.1 Swim-up

On the completion of liquefaction the semen sample was diluted 1:2 (semen: sperm washing medium- [SAGE Advantage HEPES buffered sperm preparation medium]) in a test tube and centrifuged at 450xg for 10 minutes. After centrifugation the supernatant was removed; the pellet resuspended in 2ml sperm washing medium and centrifuged in the same way. After the second centrifugation the supernatant was removed and the pellet carefully overlaid with 0.5ml sperm washing medium. This was left to stand at a 45^{o} angle for one hour at 37^{o}C. The healthy, active sperm swim up into the culture medium, leaving behind debris as well as leucocytes, dead sperm, and bacteria. As the sperm swam up to and reached this medium, they were collected by aspiration with a pipette and placed in a clean tube. This sample of 0.5ml was evaluated and was now ready for use in a fertilization/insemination procedure.

2.2.2 Sep-D Kit

The device (Surelife SEP-D Kit) contains 1ml of pre-filled semen processing medium. The cap of the device was removed from the tip and all air bubbles were removed. 1.5ml of liquefied semen was slowly aspirated while holding the device in a vertical position, to avoid mixing of the semen with the medium. The cap was then replaced and the device was kept vertically without shaking at 37^{o}C for one hour. The cap was removed and the semen was gently expelled, followed by culture medium retaining only 0.5ml of culture medium containing the motile sperm in the device (Figure 2.2).

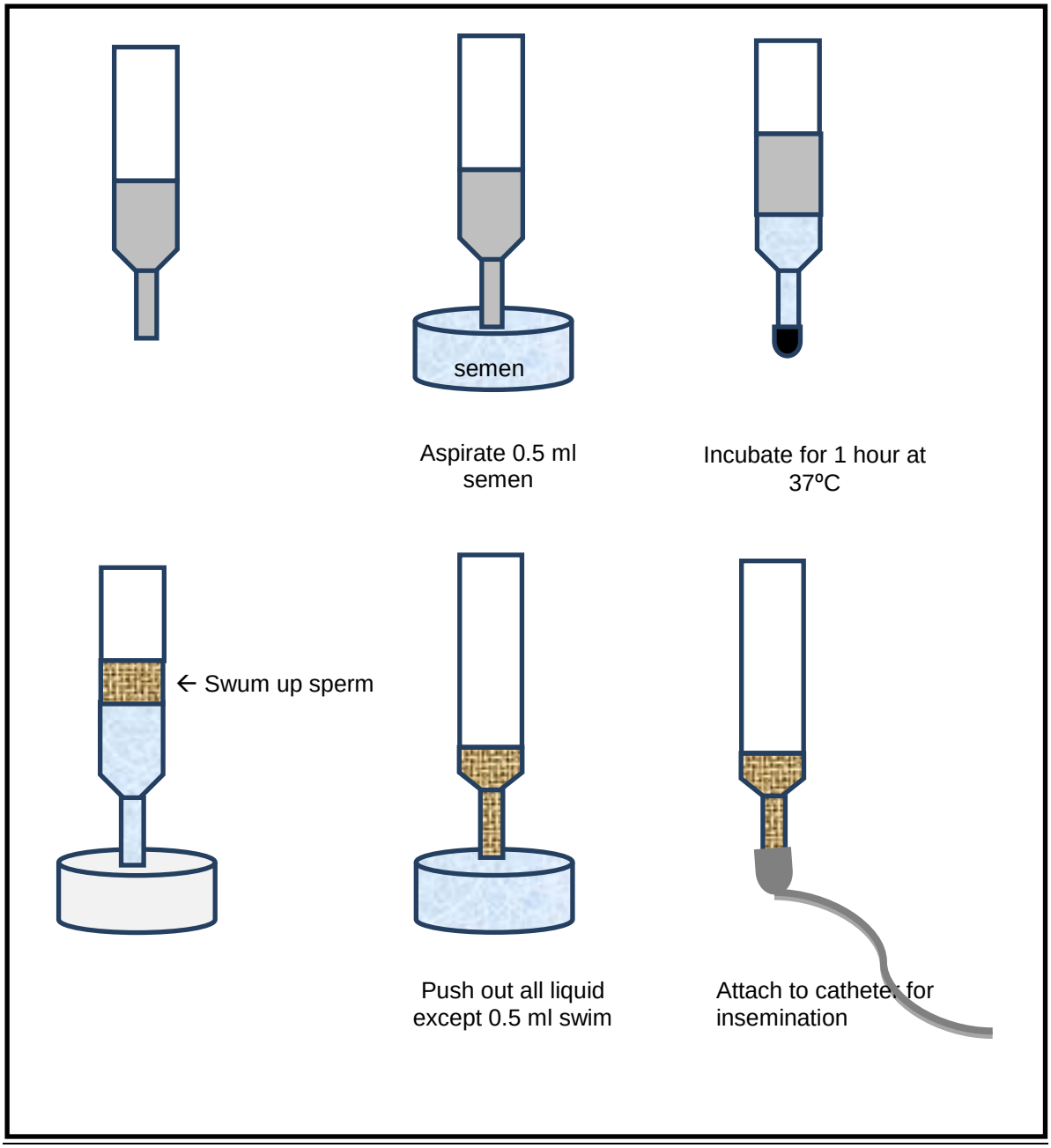


Figure 2.2 Schematic representation of the different steps in the Sep-D Kit semen preparation method

2.3 Post-preparation analysis:

A wet prep was made by placing a 10µl drop of washed sperm on a clean glass slide and covered with a 22mm x 22mm cover slip. From this sperm motility, forward progression and estimated concentration, as well as the number of cells present were determined (Appendix III).

Two smears of the washed sperm were made by applying a drop to the end of the slide. A second slide was used to pull the drop of semen along the surface of the slide (see figure 2.1 above). Slides were allowed to air dry at room temperature.

One smear was used to conduct Diff Quik staining technique and ascertain sperm morphology by following the Tygerberg Strict Criteria method (Appendix IV). The second smear was used to evaluate the chromatin packaging quality of the spermatozoa by conducting CMA_3 staining (Appendix V).

50µl sperm suspension with 150µl PBS was centrifuged for 10 minutes at 300xg. The supernatant was discarded and the pellet resuspended 150µl PBS. This process was repeated. 50µl of the sperm suspension was pipetted onto a starfrost slide and a smear was made. Slides were allowed to air dry at room temperature. The washed, air dried smear was used to conduct the TUNEL assay (Appendix VI) in order to evaluate DNA fragmentation which is a hallmark of apoptosis.

From the results obtained above calculate the Total Motile Count (TMC):

TMC= (Concentration x Motility x Volume)/100

At the Drs Aevitas Fertility Clinic, Vincent Pallotti Hospital, female patients were stimulated from day 4 to 8 with either 50mg or 100mg Clomid. Clomid may have been replaced by 5mg Femara. An ultrasound was done on day 11 of the patients' cycle and if follicles greater than 18mm were observed the patient received HCG (human chorionic gonadotropin) in order to stimulate ovulation. Insemination was performed 36 hours post HCG.

2.4 Pregnancy evaluation

Positive biochemical pregnancy in this study was taken as βhCG ≥5 ten days post IUI (βhCG is the hormone produced by the cells of the embryo once it has implanted within the endometrium.)

The study received ethical approval from the ethics committee of the faculty of medicine and Health Sciences of Stellenbosch University.

2.5 Statistical analysis

Statistical analysis was done by Dr Carl Lombard [The Biostatistics Unit (BU) of the South African Medical Research Council (MRC)]

To analyse the data several statistical models were investigated. Data was visualized using Lowess Smoother graphs. A non-inferiority analyses was performed on the two sets data (Swim-up versus Sep-D) using a binomial regression model to estimate the difference and 90% confidence interval. Bounds for inferiority were set for each

parameter. In some cases a quantile regression model was used to estimate the difference in medians between the two methods and obtaining a 90% confidence interval for the difference. Analysis of covariance [ANCOVA] was also performed using the “before preparation” data as a covariate to improve precision. This was evaluated using a linear regression model. For certain outcomes the Wilcoxon Rank test and Fisher’s exact test was also performed. Finally a univariate and multiple regression models giving odds ratios were also performed to analyse data.

CHAPTER 3

3. RESULTS

3.1 TRIAL GROUP RESULTS (n=473)

Patients were randomly assigned to either the Swim-up or the Sep-D preparation method by a randomised table (Appendix II). A total of 231 patients were assigned the swim-up method, and 242 patients were assigned the Sep-D preparation method, a total of 473 patients.

3.1.1 Descriptive data

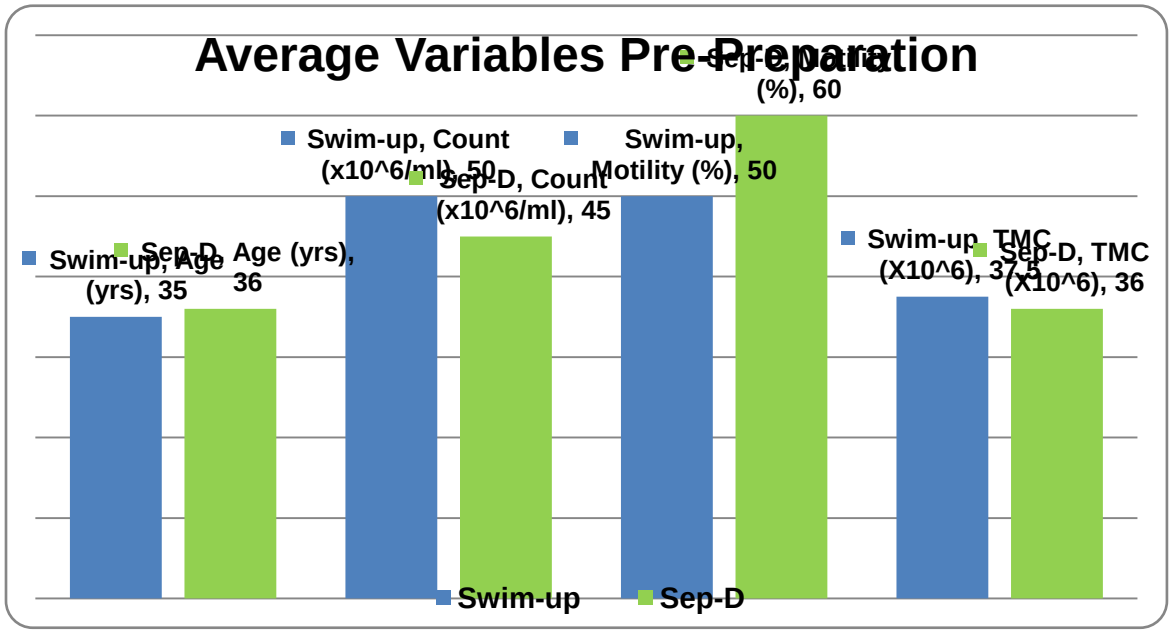


Figure 3.1 Histogram presenting the average variables of pre-preparation semen samples for the two preparation methods indicating good randomisation

Randomisation was successful in achieving comparable groups for the swim-up and Sep-D semen preparation methods (Figure 3.1).

3.1.2 Pregnancy

The IUI pregnancy outcome after insemination of Sep-D kit prepared semen was non-inferior to that of the standard swim up method (Table 3.1).

Table 3.1 Biochemical pregnancy outcomes in patients post IUI with swim-up versus Sep-D kit prepared semen samples (n=473)

	Swim-up*	Sep-D	Swim-up + Sep-D
No. Pregnancies	24	28	52
No. Patients	231	242	473
Pregnancy rate (%)	10.39*	11.57	10.99

**Outcome with 2 missing values in swim-up group*

Non-inferiority analysis for pregnancy – Swim-up versus Sep-D Kit semen preparation:

The test is conducted by calculating the lower bound for the 90% confidence interval of the difference between Sep-D and swim-up. This is testing at a 5% level of significance. Using a binomial regression model to estimate the difference and confidence level.

The estimated lower bound of the 90% confidence interval is -0.035. Since this is larger than the non-inferiority bound of -0.05 pre specified, one can conclude the non-inferiority of Sep-D in comparison to swim-up.

*Drs Aevitas Fertility Clinic is a referral clinic and often deals with overseas patients. The missing values were from two such patients in which all communication had been lost (Table 3.1).

Sensitivity analysis

1) Taking missing pregnancy outcomes both as not pregnant, the estimated lower bound of the 90% confidence interval is -0.034 which is larger than -0.05 and thus non-inferiority result still holds.
2) Taking one of the missing outcomes as pregnant and the other as non-pregnant, the estimated lower bound of the 90% confidence interval is -0.039 which is larger than -0.05 and thus non-inferiority result still holds.
3) Assuming missing outcomes both as pregnancies, the estimated lower bound of the 90% confidence interval is -0.044 which is larger than -0.05 and thus non-inferiority still holds.

Thus irrespective of the best case or worst case scenario for the participants with missing outcome data, the hypothesis of non-inferiority is accepted across all scenarios.

3.1.3 Post-preparation Count, Motility and TMC

Post preparation semen parameters for the swim-up and Sep-D Kit prepared semen samples are presented in Table 3.2.

Table 3.2 Post-preparation semen parameter distribution in the swim-up and Sep-D Kit prepared semen samples

METHOD	VARIABLE	Min	P25	P50	P75	max
Swim-up	Count (x10^6/ml)	0.5	12	25	40	100
	Motility (%)	9	90	95	95	99
	TMC (x10^6)	0.135	4.95	11.875	19	49.5
Sep-D	Count (x10^6/ml)	3	15	22	35	100
	Motility (%)	50	80	90	95	99
	TMC (x10^6)	1	6	9.9	14.85	90.1

Count (post-preparation)

The swim-up semen preparation method was non inferior for post preparation count when compared to the Sep-D Kit method

1) To test for non-inferiority a quantile regression method was used and it showed that the lower bound of the 90% confidence interval is -7.2. This exceeds the non-inferiority bound of -5.0. Hence one cannot conclude equivalence.
2) Analysis of co-variance (ANCOVA) was also performed:

   Pre-preparation count values are important predictors and inclusion in the model improves the precision for the estimate of the difference between methods. For ANCOVA (using linear regression) the lower bound is now -3.5 and hence this is larger than the non-inferiority bound of -5.0. We can therefore conclude non-

inferiority – taking pre-preparation values into account, post preparation count was not different for the two methods.

The difference in medians observed post treatment is dependent on the existing pre-value. The randomization left the Sep-D method group with lower pre-preparation values and this effect carries through to after treatment. Adjusting takes account of this difference.

Motility (post-preparation)

The swim-up semen preparation method performed superior post-preparation motility compared to the Sep-D Kit method.

1. To test for non-inferiority a quantile regression method was used and it showed that the lower bound of the 90% confidence interval is -11.6 which is smaller than the non-inferiority bound of -5.0. Hence we cannot conclude non-inferiority.
2. Analysis of co-variance (ANCOVA) was alo performed:

   Motility before is used as co-variate. Using linear regression, the lower bound of the 90% confidence interval for this method is -6.1 which is smaller than the non-inferiority bound of -0.5. Thus we cannot conclude non-inferiority. There is therefore a significant difference between the two methods. The swim-up method produces a significantly higher mean motility after preparation. The two analyses, Quantile and ANCOVA, both show evidence against concluding in non-inferiority. From the ANCOVA we can in fact conclude superiority of swim-up method over Sep-D method for motility results.

Total Motile Count (TMC) (post-preparation)

The TMC of the swim-up semen preparation method was not inferior to the TMC of the Sep-D Kit method.

1) To test for inferiority analysis of co-variance (ANCOVA) – using quantile regression was performed:

   The pre-preparation value is an important factor in determining the post-preparation value. The median difference is very small and the estimated lower bound of the 90% confidence interval is -1.03. 5% of 37.5 (the pre-preparation value of the swim-up group) is 2. The lower bound of the difference between the medians for TMC is -1.03 which is larger than -2.0, the non-inferiority bound. Thus one can conclude non-inferiority for Sep-D Kit method in terms of TMC.

Summary of primary objective outcomes

- Sep-D Kit method was **not inferior** to Swim-up method for IUI biochemical pregnancy outcome
- Sep-D Kit method was **not inferior** to Swim-up method for post-preparation count (taking pre-preparation count into consideration)
- Sep-D Kit method was **inferior** to Swim-up method for post-preparation motility (taking pre-preparation motility into consideration)
- Sep-D Kit method was **not inferior** to Swim-up method for post-preparation TCM (taking pre-preparation TCM into consideration)

3.2 SUBGROUP RESULTS (n=202)

A subgroup of 202 IUI cycles (out of the 473 cycles) was evaluated for morphology and DNA integrity results. At the Drs Aevitas Fertility Clinic various medical scientists perform the semen washing technique, thus the principle investigator was only able to capture complete data sets for 202 IUI cycles. There is however, an imbalance between the two preparation methods. 92 patients were prepared by the swim-up method while 110 patients were prepared by the Sep-D method - therefore not fully randomised.

This subgroup was analysed and compared for biochemical pregnancy, sperm DNA compaction (CMA_3) and sperm DNA fragmentation (TUNEL).

3.2.1 Pregnancy

There is a slight difference in pregnancy rates in this subgroup (Table 33).

No analysis for non-inferiority was done since a proper analysis was done on the complete trial group (n=473) above.

Table 3.3 Biochemical pregnancy outcomes in patients post IUI with swim-up versus Sep-D Kit prepared semen samples (n=202)

	Swim-up	Sep-D	Swim-up + Sep-D
No. Pregnancies	11	9	20
No. Patients	92	110	202
Pregnancy rate (%)	11.96	8.18	9.90

3.2.2 CMA_3

The post-preparation abnormal CMA_3 values are lower, in both the swim-up and Sep-D Kit samples, compared to the initial pre-preparation values. The levels and distribution are the same (Figure 3.2).

The Sep-D Kit semen preparation method is non-inferior to the swim-up method for post-preparation CMA_3.

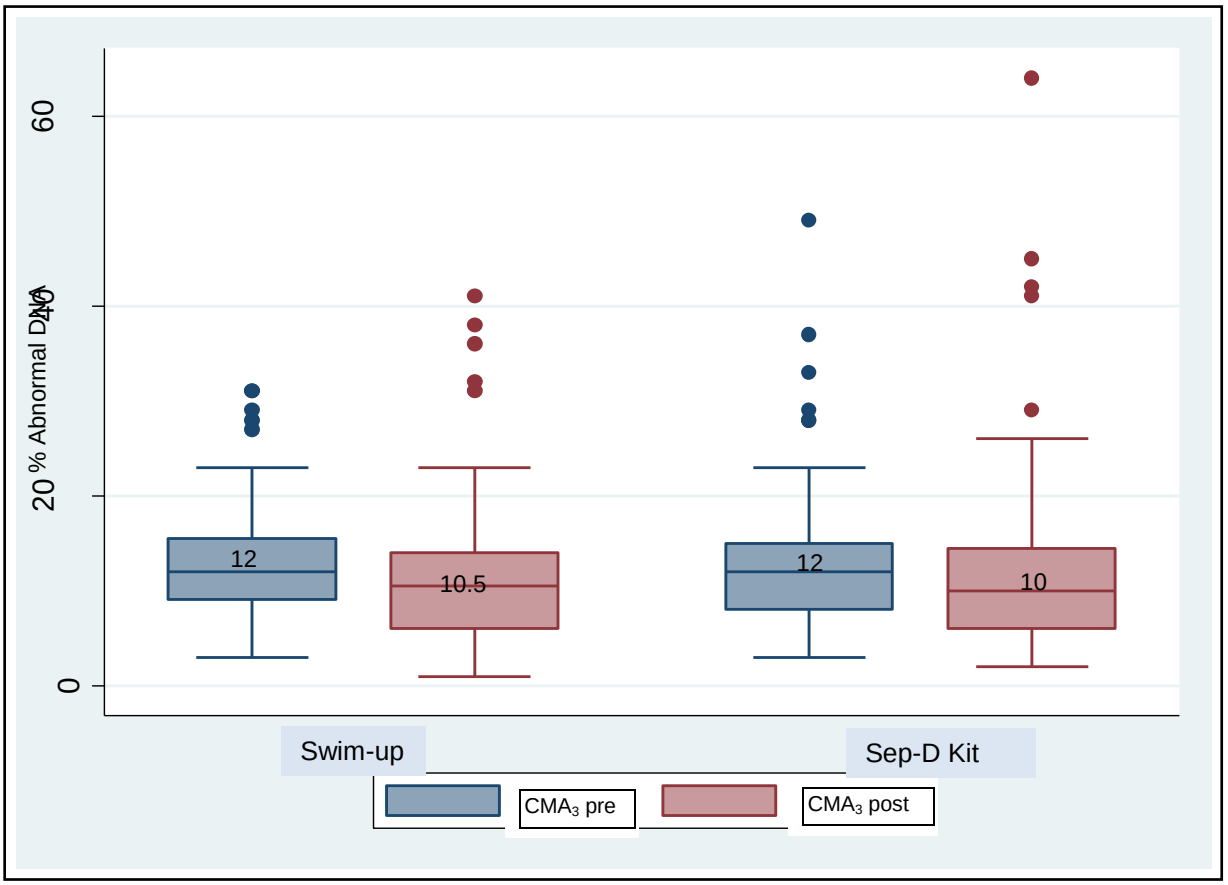


Figure 3.2 Boxplots showing the abnormal pre- versus post-preparation CMA_3 values for the swim-up and Sep–D Kit methods.

1) To test for the equivalence in CMA_3 post-preparation outcome for the two semen preparation methods, a quaintile regression method was used. The lower bound of the 90% confidence interval for this method is -2.7 which is bigger than the non-inferiority bound of -5.0. Thus we can conclude that the Sep-D Kit method is non-inferior to the swim-up method for post-preparation CMA_3.

3.2.3 TUNEL

Only post-preparation TUNEL was analysed.

Slightly higher TUNEL values (higher % abnormal DNA) was achieved after post-preparation with Sep-D versus swim-up (5% versus 4% respectively) [figure 3.3]. Sep-D Kit semen preparation method however was non-inferior to the swim-up method with regards to post preparation DNA fragmentation (TUNEL).

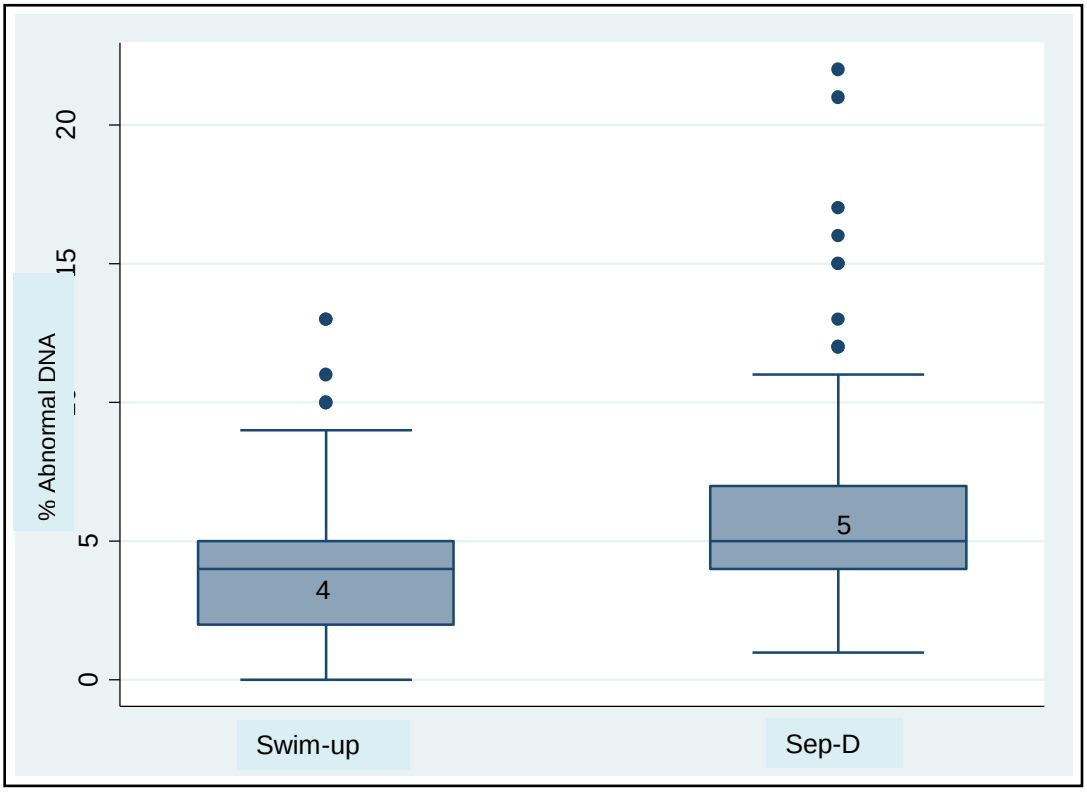


Figure 3.3 Boxplots showing abnormal post-preparation TUNEL values of swim-up versus Sep-D Kit semen preparation methods

To test for the equivalence in CMA_3 post-preparation outcome for the two semen preparation methods, a quaintile regression method was used. The lower bound of the 90% confidence interval is -0.63 which is greater than the non-inferiority bound

of -5.0 Thus we can conclude Sep-D Kit method was non-inferior to the swim-up method with regards to post preparation TUNEL.

3.3 THE ROLE OF MALE AND FEMALE VARIABLES ON IUI BIOCHEMICAL PREGNANCY OUTCOMES

For this analysis, the two semen preparation methods were ignored and both swim-up and Sep–D Kit IUI cycles were included. Only in cases where an association is seen (Figure 3.6, 3.9, 3.11) are the semen preparation methods displayed separately to prove that the association was not due to either of the preparation methods. The variables included in this analysis were: endometrial thickness, number of follicles, female age, post preparation sperm count, motility, normal morphology, TMC, abnormal CMA_3 and TUNEL.

3.3.1 Endometrial thickness

Endometrial thickness, using the Odds ratio analysis, was not a significant predictor of pregnancy rates, p=0.354. However, no biochemical IUI pregnancies were achieved if the endometrial lining was thinner than 7mm and thicker than 11mm (Figure 3.4).

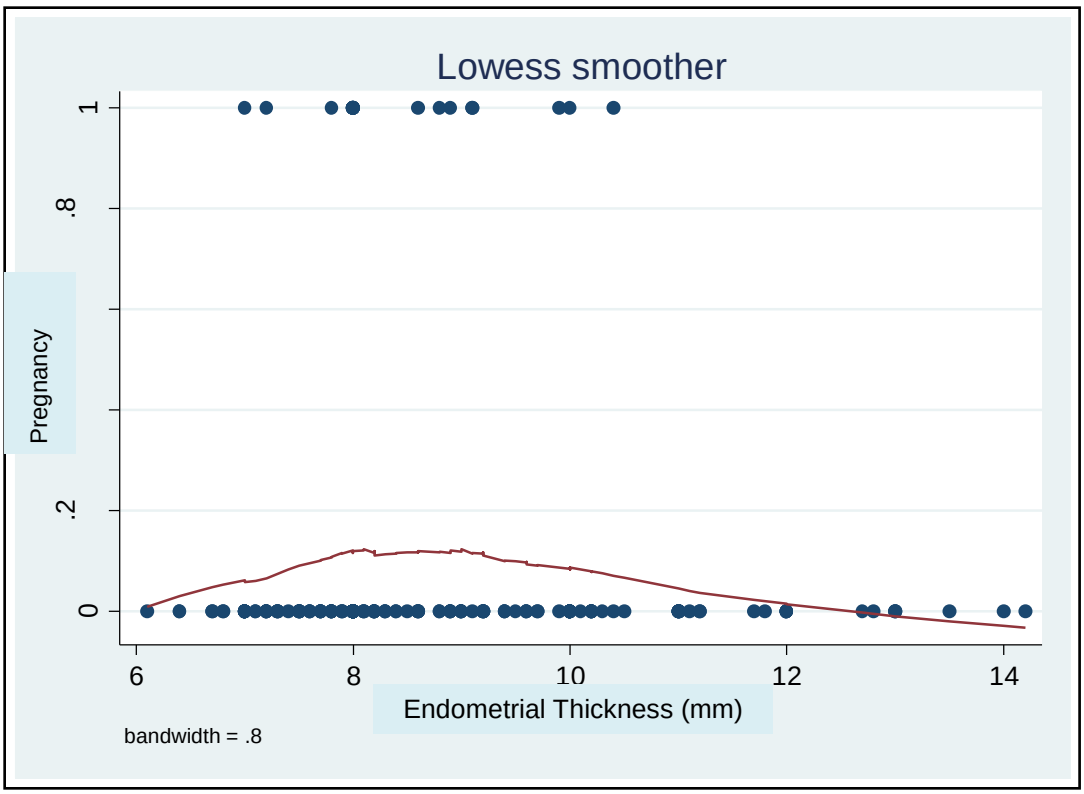


Figure 3.4 Lowess smoother graph showing the distribution of biochemical IUI pregnancies for different endometrial thicknesses (1=pregnant- top of graph versus 0=not pregnant- bottom of graph).

3.3.2 Number of follicles

The Fisher's exact test outcome showed that there is no association between the number of follicles and IUI biochemical pregnancy outcome in this dataset, p=0.828. Odds ratio analysis also showed no association, p=0.5.

3.3.3 Female Age

The biochemical pregnancy rate declines with an increase in female age. Age is a well-known risk factor and also significantly related to IUI biochemical pregnancy outcome in this data set (Figure 3.5).

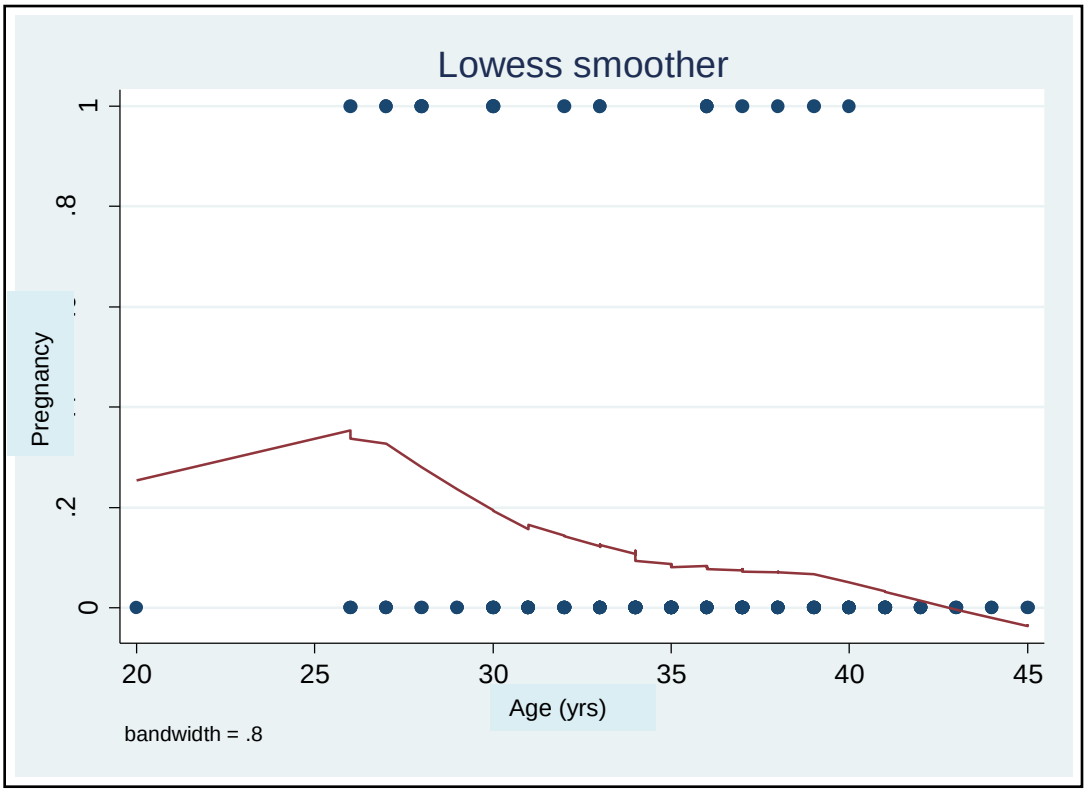


Figure 3.5 Lowess smoother graph showing the distribution of biochemical IUI pregnancies for different female ages (1=pregnant- top of graph versus 0=not pregnant- bottom of graph).

Using the odds ratio method, the odds ratio is 0.87 for every increasing year of age, decreasing the probability for pregnancy, p=0.005. The highest age of a female that became biochemically pregnant is 42 years.

3.3.4 Post-preparation count

Post-preparation count had no effect on biochemical pregnancy outcome for both the Sep-D Kit and the swim-up methods (Figure 3.6).

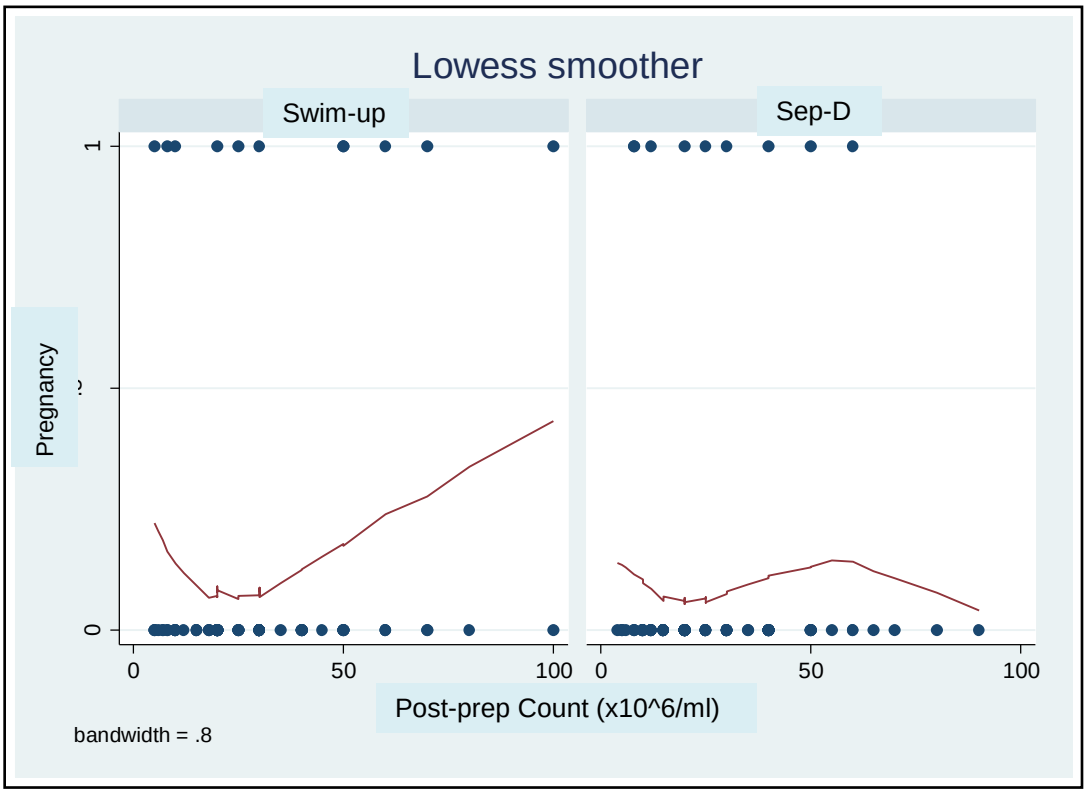


Figure 3.6 Lowess smoother graph showing the distribution of biochemical IUI pregnancies for different post-preparation semen counts (1=pregnant- top of graph versus 0=not pregnant- bottom of graph) for both swim-up and Sep-D Kit preparation methods.

Using the Odds ratio method, post-preparation count had no effect on biochemical pregnancy outcome, p=0.105. The U-shape association is seen with both preparation methods, swim-up and the Sep-D Kit. We can therefore conclude that this U-shape is not associated with the preparation method.

3.3.5 Post-preparation motility

No pregnancies were achieved with motility less than 80%.Ppost-preparation motility had a significant effect on IUI biochemical pregnancy outcome and motility clearly plays a role in affecting biochemical pregnancy outcomes and is therefore an important contributing factor (Figure 3.7).

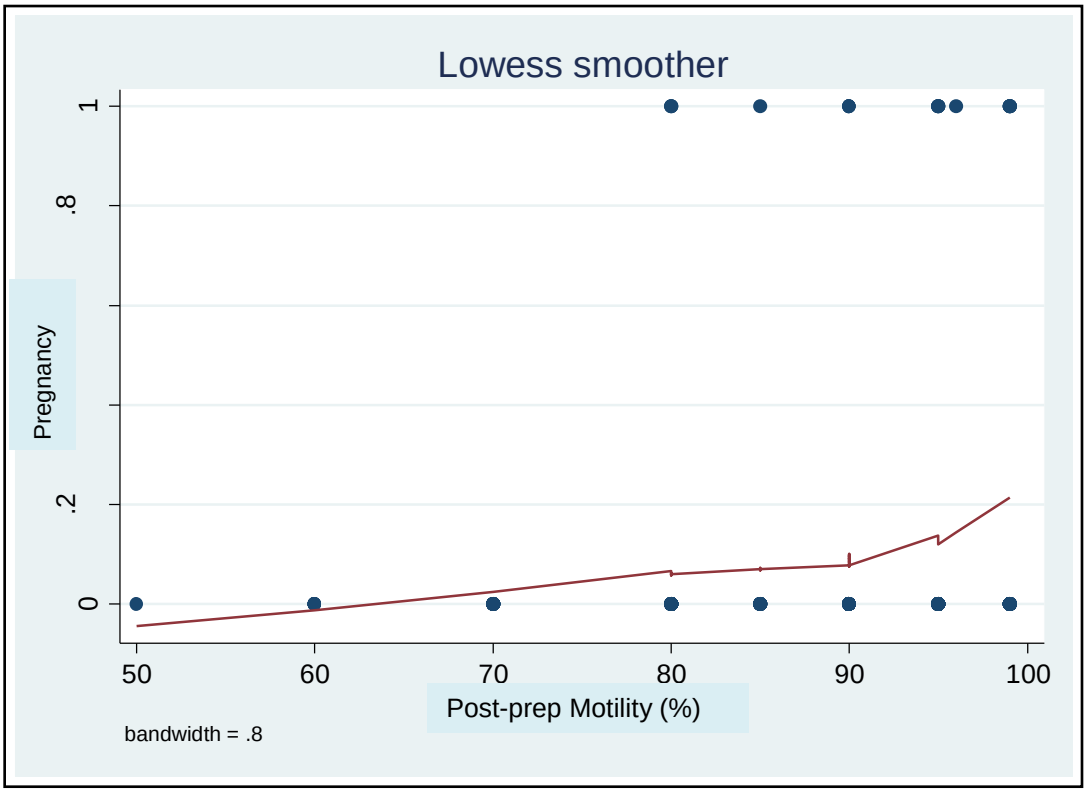


Figure 3.7 Lowess smoother graph showing the distribution of biochemical IUI pregnancies for different post-preparation motilities (1=pregnant- top of graph versus 0=not pregnant- bottom of graph).

Using the Odds ratio method, post-preparation motility had a significant effect on IUI biochemical pregnancy outcome. Odds ratio was 1.08 for every unit change in motility; p=0.036.

3.3.6 Pre- and Post-preparation morphology

1) Pre-preparation morphology

No association was found between pre-preparation morphology and IUI biochemical pregnancy outcome; (p=0.77).

2) Post-preparation morphology

There was also no association between post-preparation morphology and IUI biochemical pregnancy outcome (Figure 3.8).

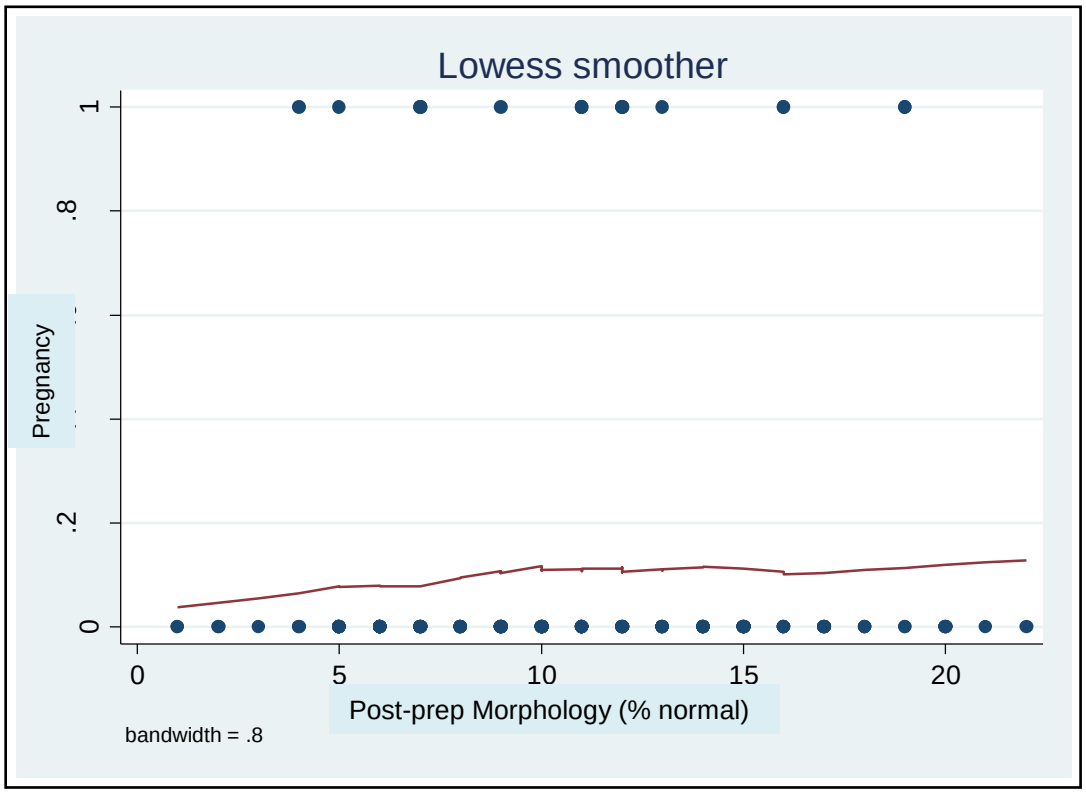


Figure 3.8 Lowess smoother graph showing the distribution of biochemical IUI pregnancies for different post-preparation sperm morphology values (1=pregnant- top of graph versus 0=not pregnant- bottom of graph).

No pregnancies were achieved when the morphology was less than 4%. The sample size in this low morphology group was small however, 8 out of 202. Therefore this association is to be expected. Using the Odds ratio model there was no association

between post-preparation morphology and IUI biochemical pregnancy outcome, p= 0.52.

3.3.7 Post-preparation Total Motile Count (TMC)

There was no association between post-preparation TMC and IUI biochemical pregnancy outcome for both the swim-up and the Sep-D Kit method (Figure 3.9).

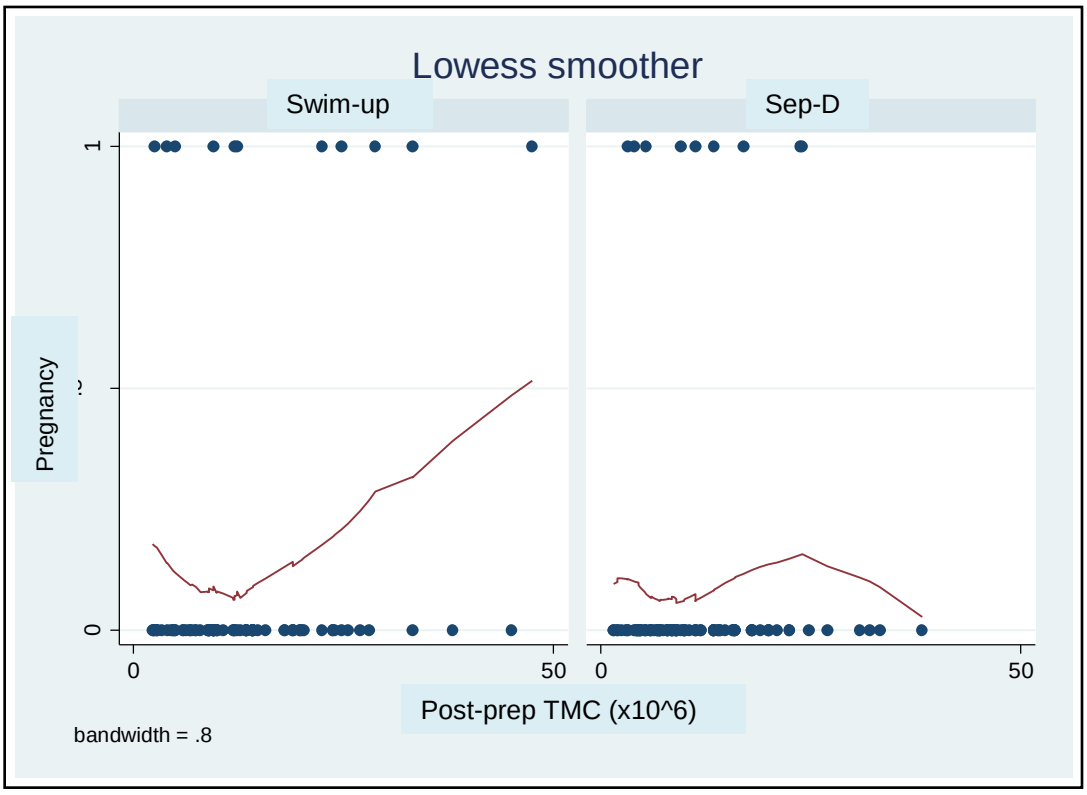


Figure 3.9 Lowess smoother graph showing the distribution of biochemical IUI pregnancies for different post-preparation TMC values (1=pregnant- top of graph versus 0=not pregnant- bottom of graph) for both swim-up and Sep-D Kit preparation methods.

The U-shape association is seen with both preparation methods, swim-up and the Sep-D-Kit. We can therefore conclude that this U-shape is not associated with the preparation method. Using the Odds ratio model there was no

association between post-preparation TMC and IUI biochemical pregnancy outcome, p= 0.054.

3.3.8 Post-preparation CMA_3

Over all there was no association between post-preparation CMA_3 and IUI biochemical pregnancy outcome.

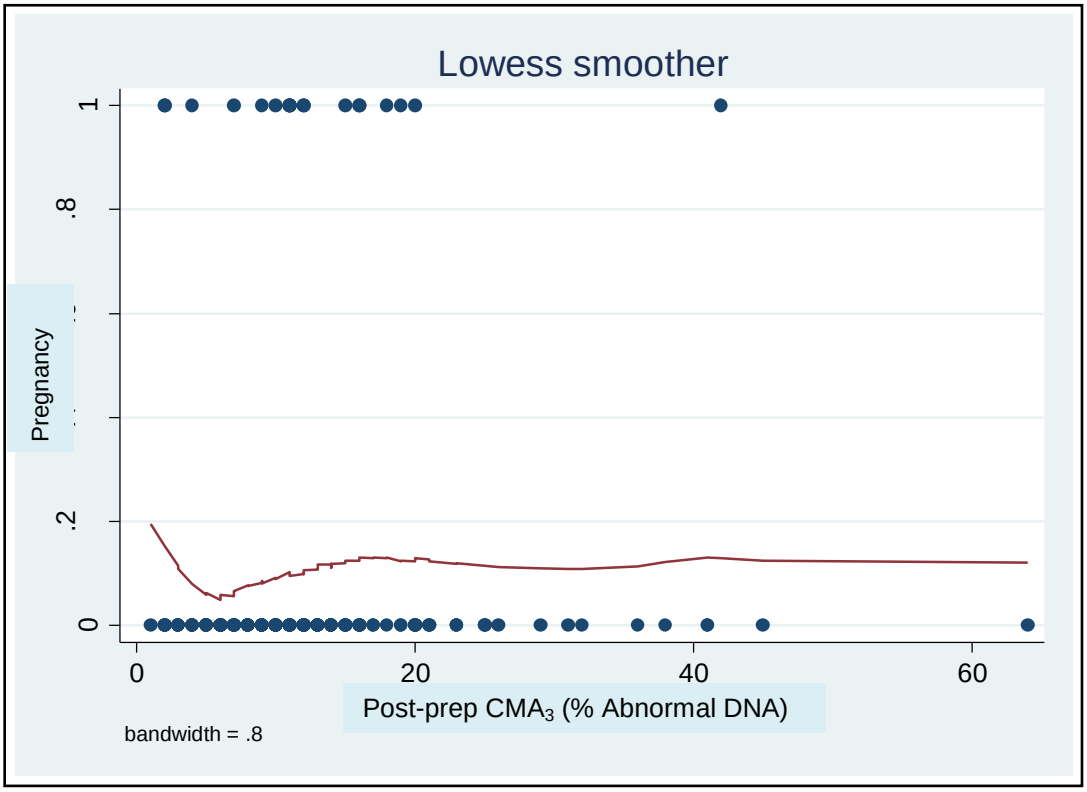


Figure 3.10 Lowess smoother graph showing the distribution of biochemical IUI pregnancies for different abnormal post-preparation CMA_3 values (1=pregnant- top of graph versus 0=not pregnant- bottom of graph).

There is an increase in pregnancies up until 15% abnormal DNA and then pregnancies remain constant. Using the Odds ratio model, over all there was no association between post-preparation CMA_3 and IUI biochemical pregnancy outcome, p= 0.57.

3.3.9 Post-preparation TUNEL

There was a significant association between post-preparation TUNEL and IUI biochemical pregnancy outcome (Figure 3.11). After 6% abnormal DNA there are no pregnancies and this is seen in preparation methods, the swim-up and the Sep-D Kit method.

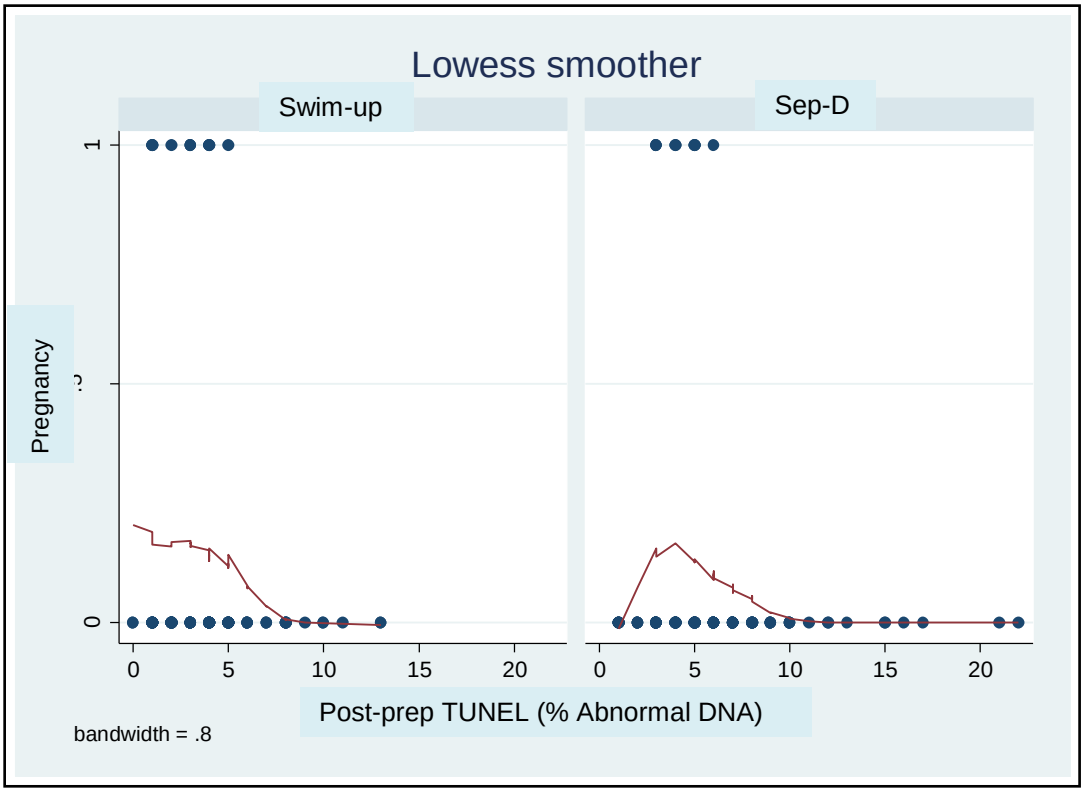


Figure 3.11 Lowess smoother graph showing the distribution of biochemical IUI pregnancies for different abnormal post-preparation TUNEL values (1=pregnant– top of graph versus 0=not pregnant- bottom of graph) for both swim-up and Sep-D Kit preparation methods.

Using the Odds ratio model, and including both methods, there was a significant association between post-preparation TUNEL and IUI biochemical pregnancy outcome: Odds ratio=0.78 for every unit change in post-preparation TUNEL, p=0.018.

3.4 MULTIPLE REGRESSION MODELS COMPARING VARIABLES AND IUI BIOCHEMICAL PREGNANCY OUTCOMES

Several models were investigated and the best one was where female age, post-preparation motility, post-preparation count and post-preparation CMA_3 was included.

For female age the odds ratio was 0.87 decreasing the probability for biochemical pregnancy for every year older (starting from age 20). The odds ratio for IUI pregnancy was a 1.11 increasing probability with higher post-preparation motility (greater than 80%).

The significant effect of female age was consistent throughout all models; post-preparation motility also did well in a number of the models. However, sperm DNA factors did not really feature as significant.

Summary of secondary objective outcomes

- Sep-D was **non-inferior** to swim-up with regards to post-preparation CMA_3
- Sep-D was **non-inferior** to swim-up with regards to post-preparation TUNEL
- Endometrial thickness was **not significant** in IUI biochemical pregnancy outcome
- The number of follicles was **not significant** in IUI biochemical pregnancy outcome
- Female age was a **significant** factor in IUI biochemical pregnancy outcome

- Post-preparation count was **not significant** in IUI biochemical pregnancy outcome
- Post-preparation motility was **significant** in IUI biochemical pregnancy outcome
- Post-preparation morphology was **not significant** in IUI biochemical pregnancy outcome
- Post-preparation TMC was **not significant** in IUI biochemical pregnancy outcome
- Post-preparation CMA_3 was **not significant** in IUI biochemical pregnancy outcome
- Post-preparation TUNEL was **significant** in IUI biochemical pregnancy outcome

- In multiple regression analysis only female age and post-preparation motility showed consistent significance in IUI biochemical pregnancy outcome

CHAPTER 4

4. DISCUSSION

Ultimately the aim of this study was to test a new IUI semen preparation method, Sep-D Kit, against the routine conventional method, swim-up, and to determine whether or not they are comparable with regards to biochemical pregnancy rates. The biochemical pregnancy rates were determined to be similar for the two methods (11.57% for swim-up and 10.39% for Sep-D) and it was found that the Sep-D is non-inferior to the swim-up method. This is the expected result as both methods have previously been proven to be comparable between post-preparation parameters, and share the same principle whereby the sperm swim up and into medium that is then collected for insemination.

The Sep-D is also non-inferior to the swim-up method with regards to post-preparation count and total motile count. The TMC is an indication of the total number of motile spermatozoa in the sample available for insemination and this is significant when comparing the two samples. These results are also to be expected as they were previously proven to be comparable in a pilot study. It was hypothesized that the Sep-D Kit method would compare well with the swim-up method regarding these parameters and our hypothesis has been proven correct.

The Sep-D Kit method is however inferior to the swim-up method with regards to post-preparation motility. This study has proven that the swim-up method produces more motile sperm post-preparation than the Sep-D Kit method. The one possible way of obtaining more non-motile spermatozoa in a prepared sample is by unintentional mixing of the semen and medium. This proves that it is thus easier for unintentional mixing of semen and medium during semen preparation by use of the Sep-D Kit method, even

when being extremely cautious. It is also possible that contamination occurs with some dead sperm still present in the syringe (Sep-D Kit) as the operator expels the final 0.5 ml out. With the swim-up method this is not possible as one is not supposed to come into close contact with the pellet.

The DNA packaging quality (CMA_3) was assessed before and after both preparation methods. The percentage abnormal DNA decreased slightly after preparation yet there was no significant difference between the two preparation methods. This means that neither preparation method selected to a better degree for spermatozoa with mature DNA and the Sep-D Kit method is therefore comparable to the swim-up method. This could once again be attributed to the fact that both preparation methods rely on the same principle of sperm swimming up and into the medium for insemination.

The DNA fragmentation (TUNEL) was only assessed post-preparation. There was no difference in the percentage fragmented DNA between the two preparation methods and Sep-D-Kit method is therefore comparable to the swim-up method. This could also be justified by reasoning that both methods rely on the same swim up principle.

Ricci *et al.* (2009) compared the standard swim-up method with the gradient-density centrifugation method and analysed certain parameters post-preparation including viability, total motile count, and motility. The article reported that both semen preparation methods obtain a sperm population with a lower percentage of apoptotic sperm compared with the original semen sample. Neither sample produced significantly different results regarding total motile count and motility. It was concluded that an ideal semen preparation method probably does not exist and therefore the method chosen

should be based on the pre-preparation semen parameters and which assisted reproductive technique is being performed.

Morshedi *et al.* (2003) agree with Ricci *et al.* (2009) that the semen preparation method chosen should be based on the pre-preparation parameters. The articles reported that the swim-up method is best used for samples with a higher number of motile sperm, and that the gradient-density centrifugation method should be used to prepare poor quality semen samples. These two studies also found no significant difference in pregnancy rates between the two semen washing methods.

Many factors, male and female, were compared against biochemical pregnancy outcome to test which significantly influence the outcome. Each factor will be discussed separately.

Female Factors

Although no pregnancies where achieved when the endometrial lining was thinner than 6mm or thicker than 11mm, endometrial thickness as a whole was not significantly associated with pregnancy results. The endometrial thickness is therefore a factor that does not affect the biochemical pregnancy outcome in our study population. A study by Esmailzadeh and Faramarzi, (2007) has shown that the endometrial thickness can be considered a predictor of pregnancy, since endometrial proliferation is needed for successful implantation. Tomlinson *et al.* (1996) found similar results and agrees that endometrial thickness is a significant variable that predicts IUI pregnancy outcome.

No association was found between the number of follicles and the biochemical pregnancy outcome in our study. Zadehmodarres *et al.* (2009) reported similar results showing that the number and size of the follicle did not have any relation to the IUI pregnancy rate. Esmailzadeh and Faramarzi (2007) as well as Nuojua-Huttunen *et al.* (1999), and Tomlinson *et* al. (1996) demonstrated differing results whereby the number of follicles and the diameter of the dominant follicle were significantly associated with IUI pregnancy outcome.

The biochemical pregnancy rate decreased with decreasing female age. This study found that female age was significantly associated with pregnancy outcome. Female age is a well-known and well documented factor affecting pregnancy outcome (Brzechffa *et* al., 1998; Nuojua-Huttunen *et al*., 1999; Montanaro Gauci *et al*., 2001; Zadehmodarres *et al*., 2009). Aging is associated with progressive follicular depletion and diminished oocyte quality. The negative impact it has on treatment may be due to the higher rate of aneuploidy found in oocytes (Esmailzadeh and Faramarzi, 2007). Although not all have found significant evidence that age predicts pregnancy rates, it is agreed that increasing age has negatively impacts on pregnancy (Esmailzadeh and Faramarzi, 2007; Dovey *et al.,* 2008).

Male Factors

Post-preparation count had no significant effect on biochemical pregnancy outcome; however the pregnancy rate does increase with increasing count. As the number of sperm increase, the chances of fertilization occurring increases and therefore so does the pregnancy rate. No pregnancies where achieved with a count of less than 5million

spermatozoa/ml. Many articles have reported similar findings to this study, whereby although sperm count is not necessarily a significant predictor of pregnancy, it has shown to have a direct influence on the outcome (Shulman *et al*., 1998; Zadehmodarres *et al*., 2009; Badawy *et al*., 2009).

Post-preparation motility had a significant association with biochemical pregnancy outcome. No pregnancies were achieved with a motility less that 80%. This proves that there needs to be a significant amount of motile sperm in the sample in order for the chances of fertilization to take place and therefore positively affect the chances of conception. Various studies reported similar results that motility is a significant predictor of pregnancy (Tomlinson *et al*., 1996; Shulman *et al*., 1998; van der Westerlaken *et al*., 1998; Montanaro Gauci *et al*., 2001; Esmailzadeh and Faramarzi, 2007).

Post-preparation total motile count (TMC) had no significant effect on biochemical pregnancy outcome. Most articles state that TMC is a significant predictor of pregnancy, however their values all differ. In 1996 Campana *et al*. found that a TMC of $>1x10^6$ is necessary for an IUI pregnancy, while Miller *et al*. (2002) found that a TMC of $\geq 10x10^6$ is necessary. Merviel *et al.* (2010) describes the best chance of an IUI pregnancy with a sample having a TMC of $\geq 5x10^6$ spermatozoa. Although TMC had no significant effect on biochemical pregnancy, it was noted that pregnancy rates increased when samples had a TMC $\geq 10x10^6$. However, pregnancies were also achieved with a TMC as low as $2x10^6$.

Post-preparation morphology had no significant effect on biochemical pregnancy outcome; however no pregnancies were achieved when the percentage normal forms was less than 5. Although this seems important it is not significant and this result may

have been due to the small sample size (n=8) of patients with less than 5% normal sperm morphology. Many articles disagree with this result and state that morphology is a significant predictor of pregnancy (Toner *et al*., 1995; Van Waart *et al*., 2001; Montanaro Gauci *et al*., 2001; Hauser *et al*., 2001; Ombelet *et al*., 2003).

The post-preparation DNA packaging quality (CMA_3) had no significant effect on biochemical pregnancy outcome. The pregnancy rate increases with increasing percentage abnormal DNA but then stays constant from 15% abnormal DNA onwards.

The post-preparation DNA fragmentation (TUNEL) also had no significant effect on biochemical pregnancy outcome when all parameters where taken into consideration; however no pregnancies were achieved when more than 6% abnormal DNA was present. Duran *et al.* (2002) proved that sperm DNA quality predicts intrauterine insemination pregnancy outcome. No samples with >12% of sperm having DNA fragmentation resulted in pregnancy.

In conclusion, the only female parameter that significantly predicts biochemical pregnancy outcome is age and this has been well documented throughout the literature. Motility (post-preparation) is the only male parameter that significantly affects biochemical pregnancy outcome. No pregnancies were achieved when the motility was <80%.

The Sep-D Kit method is non-inferior to the swim-up method with regards to biochemical pregnancy rates, post-preparation count and TMC. The swim-up method produces samples with a significantly higher post-preparation motility compared to the Sep-D Kit method, however they still manage to produce similar pregnancy rates. The

Sep-D Kit method may even be the better method to use for IUI, as it is simple, fast and effective. Although the Sep-D Kit may be rather expensive at first glance, there are 5 devices (containing sperm washing medium) in a Kit, and the insemination catheter is also provided. No expensive laboratory equipment or embryologist is needed to prepare the spermatozoa, it is time saving, and has proven to be comparable to the standard swim-up preparation method. Overall the Sep-D Kit method is cheaper than the standard swim-up method (R242.10 and R334.03 respectively), it is more time efficient (2hours needed to perform a swim-up whereas only 1hour necessary for a Sep-D Kit), and the method is easier to perform. The Sep-D Kit may therefore be used as a standard semen preparation method, and it may be implemented in developing countries for use in routine IUI procedures. A central question remains however, can this relatively expensive technique which has a 10.39% success rate (biochemical pregnancy rate) be justified in countries where poverty is still an important issue. It is obvious that infertility treatment in developing countries still requires great attention at both a National and International level (Ombelet *et al*., 2008).

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APPENDICES

APPENDIX I

IUI INFORMATION FORM

Date: _____

Patient #: _____

Sperm Preparation Method: _____

Male parameters:

Semen parameters	**Initial analysis Pre-preparation**	**Post analysis Post Preparation**
Time passed		
Time analysed		
Abstinence (days)		
Volume (ml)		100µl
Viscosity (cm)		
Cells		
Concentration ($x10^6$/ml) [estimation]		
Motility (%)		
Forward Progression		
Morphology (% normal)		
CMA_3 (% abnormal)		
Tunel (% abnormal)		
Total Motile Count ($x10^6$ml)		
Remarks		

Female parameters:

Age of female	
Cycle #	
Diagnosis	
# follicles	
Endometrium thickness (mm)	
Pregnancy outcome	

APENDIX II

RANDOMISED TABLE

Randomised Table 1-25

	Method	NAME	DOB	+/-
1	A			
2	A			
3	B			
4	B			
5	B			
6	A			
7	B			
8	A			
9	A			
10	A			
11	B			
12	B			
13	A			
14	A			
15	A			
16	B			
17	B			
18	A			
19	B			
20	B			
21	A			
22	B			
23	A			
24	A			
25	B			

A Swim-up
B Sep-D

APPENDIX III

ROUTINE SEMEN ANALYSIS (WHO, 1999)

Semen viscosity

After liquefaction, the viscosity of the sample can be estimated by gently aspirating it into a wide-bore (approximately 1.5 mm diameter) plastic disposable pipette, allowing the semen to drop by gravity and observing the length of any thread. A normal sample leaves the pipette in small discrete drops. If viscosity is abnormal, the drop will form a thread more than 2 cm long.

Semen volume

The volume of the ejaculate is contributed mainly by the seminal vesicles and prostate gland, with a small amount from the bulbourethral glands and epididymis. Precise measurement of volume is essential in any evaluation of semen, because it allows the total number of spermatozoa and non-sperm cells in the ejaculate to be calculated. The volume can be measured directly from a test tube.

Semen pH

The pH of semen reflects the balance between the pH values of the different accessory gland secretions, mainly the alkaline seminal vesicular secretion and the acidic prostatic secretion. The pH should be measured after liquefaction at a uniform time, preferably after 30 minutes, but in any case within one hour of ejaculation since it is influenced by the loss of CO_2 that occurs after production.

For normal samples, pH paper in the range 6.0 to 10.0 should be used.

1. Spread a drop of semen evenly onto the pH paper.
2. Wait for the colour of the impregnated zone to become uniform (< 30 seconds) and compare it with the calibration strip to read the pH.

Wet preparation

One drop of semen (10µl) was placed onto a clean glass slide and covered with a coverslip (22mm × 22mm). The weight of the coverslip should spread the sample

evenly. Care was taken in order to avoid the formation and trapping of air bubbles between the coverslip and the slide. The freshly made wet preparation was assessed as soon as the contents are no longer drifting, using a regular light microscope at a 400x magnification.

Motility

In determining quantitative motility one distinguishes the percentage of motile spermatozoa from the percentage of immotile spermatozoa. The estimation of the percent motile is made to the nearest 10 percent (Menkveld and Coetzee, 1995).

Forward progression

In determining qualitative motility, in our laboratory, the nature of the motility is evaluated on a scale of 0 to 4.

0 No movement
1 Movement (twitching)-none forward
1+ Movement-every now and then
2 Movement-undirected and slow
2+ Movement-slowly but directly forward
3- Movement-fast but not direct
3 Movement-fast and direct
3+ Movement-very fast and direct
4 Movement-extremely fast and direct

Cells

Somatic cells (leukocytes, histocytes and epithelium) were observed on the slide (at 400x magnification) and expressed as follows:

± A few cells on the slide
+ 1-5 cells per high power field
++ 6-10 cells per high power field
+++ >10 cells per high power field

APPENDIX IV

DIFF QUIK MORPHOLOGY STAINING

1. Dip in solution 1 (fixative) for 10 seconds.
2. Dip in solution 2 (red) for 7 seconds.
3. Dip in solution 3 (purple) for 7 seconds.
4. Rinse in tap water, 4 dips.
5. Air dry.
6. Mount sections in DPX.

The fixative is triarylmethane, while solution 2 and 3 are xanthene and thiazine respectively.

APPENDIX V

CMA_3 STAINING AND EVALUATION

Method

1. Place in methanol:acetic acid (3:1) fixative for 20 min at room temperature.
2. Slides are air dried and stained with 60µl CMA_3.
3. Place stained slides in a dark chamber for 20 minutes.
4. Rinse slides in McIlvaines buffer and immediately mount.

Mounting

1. Use Dabco to mount slides.
2. Place wet slide on fume hood table.
3. Add 2 drops of Dabco on the slide.
4. Hold one end of the cover slip on the Dabco and allow the Dabco to disperse.
5. Thereafter drop entire cover slip on slide.
6. Check for air bubbles and gently remove.

Evaluation

1. Slides are evaluated using a fluorescence microscope which should be switched on at least 20 minutes prior to usage.
2. Place the slide onto the stage of the microscope.
3. Find a field that has an evenly dispersed, surplus amount of spermatozoa under 20 X magnification (Phase Contrast).
4. Place a drop of immersion oil on the specific field and change to the 100 X phase contrast objective.
5. Focus and count 100 spermatozoa.
6. The spermatozoa should be counted by the degree of fluorescence on the sperm head.
7. These are classes of sperm to look for:
   - No Staining (No fluorescence)
   - Fluorescence band at equatorial segment
   - Fluorescent stain around periphery of head (did not permeate membrane).
   - Fluorescent staining (faintly yellow)
   - Bright yellow fluorescent staining
8. The first three classes are indicative of good quality packaging DNA in the sperm head and are regarded as CMA_3 negative.
9. The last two classes are indicative of poor packaging DNA in the sperm head and regarded as CMA_3 positive.
10. The spermatozoa should be counted as CMA_3 negative versus CMA_3 positive.
11. Report the number, as a percentage, of CMA_3 positive spermatozoa.

APPENDIX VI

TUNEL ASSAY AND EVALUATION

TUNEL Assay

Do step 7 while waiting for the other steps 1-6.
Fold edges of plastic cover slips before use.

1. Fix sperm by immersing slides in freshly prepared 4% formaldehyde in PBS for 25 minutes.
2. Wash slides carefully in fresh PBS for 5 minutes at room temperature.
3. Permeabilize cells in 0.2% Triton X-100 in PBS for 5 minutes.
4. Rinse slides twice in fresh PBS for 5 minutes at room temperature. (1x PBS for 5 minutes and 1x fresh PBS for 5 minutes)
5. Remove excess liquid by tapping slides on paper towel.
6. Add 100µl of equilibration buffer (bottle in kit) onto each slide and equilibrate cells for 5 minutes.
7. Thaw the nucleotide mix (eppi in kit) and prepare sufficient TdT incubation buffer. (Make the buffer in an eppi and store in foil.)

Buffer component	Component volume per slide		Number of slides		Component volume
Equilibration buffer	18 µl	X	*18*	=	*324 µl*
Nucleotide mix	2 µl	X	*18*	=	*36 µl*
TdT enzyme	0.4 µl	X	*18*	=	*7.2 µl*

Total TdT incubation buffer volume = *367.2 µl*

8. Blot slides on paper towel and add 20µl of TdT incubation buffer to the cells. Do not allow the cells to dry out!
9. Cover the cells with plastic cover slips. Incubate slides at 37°C for 60 minutes inside the humidifying chamber.
10. Remove the plastic cover slips and terminate the reaction by immersing the slides in 2x SSC for 15 minutes at room temperature.
11. Wash slides in d.H_2O for 5 minutes at room temperature in a dark chamber. Repeat 2 times.
12. Drain off excess water from the slides.
13. Analyze slides immediately.

Evaluation

1. Slides are evaluated using a fluorescence microscope which should be switched on at least 20 minutes prior to usage.
2. Place the slide onto the stage of the microscope.

3. Find a field that has an evenly dispersed, surplus amount of spermatozoa under 20 X magnification (Phase Contrast).
4. Place a drop of immersion oil on the specific field and change to the 100 X phase contrast objective.
5. Focus and count 100 spermatozoa.
6. The spermatozoa should be counted by the degree of fluorescence on the sperm head.
7. These are classes of sperm to look for:
   No Staining (No fluorescence)
   Fluorescence band at equatorial segment
   Fluorescent stain around periphery of head (did not permeate membrane)
   Fluorescent staining (faintly green or patchy)
   Bright green fluorescent staining
8. The first three classes are indicative of good quality DNA with no fragmentation in the sperm head and are regarded as TUNEL negative.
9. The last two classes are indicative of poor quality DNA in the sperm head, a result of fragmentation, and are regarded as TUNEL positive.
10. The spermatozoa should be counted as TUNEL negative versus TUNEL positive.
11. Report the number, as a percentage, of TUNEL positive spermatozoa.