

**A comparison of motility and head  
morphology of sperm using  
different semen processing methods  
and three different staining  
techniques**

by

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## DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature:.....

Date:.....

## ABSTRACT

Sperm morphology remains an important parameter in the prediction of fertility, both *in vivo* and *in vitro*. However, there remains a considerable level of concern surrounding the true potential of this parameter due to the lack of standardization of differential staining techniques used for the evaluation of sperm morphology. This study aimed at investigating two commonly used staining techniques, Rapidiff<sup>®</sup> (RD) and Papanicolaou (PAP), along with a new commercially available stain, SpermBlue<sup>®</sup> (SB), in the evaluation of sperm morphometry and morphology. Results indicated that significant differences in sperm morphometry exist due to the use of the staining techniques. Findings further indicated that RD causes sperm head swelling while PAP causes sperm head shrinkage. Results obtained using the SB staining technique have indicated measurements closest to that which would be obtained through the evaluation of fresh, unstained sperm. The lack of standardization and the different effects various stains have on sperm structure and overall sperm morphology evaluation should raise a level of concern, particularly when evaluating patients with borderline morphology. Based on this, the use of the SB staining technique is recommended over RD and PAP for effective and accurate morphology evaluation. In further support of this technique, SB was shown to be quick and simple in method, and allowed for the easy detection of sperm by computer aided sperm analysis (CASA) systems such as the Sperm Class Analyzer (SCA<sup>®</sup>).

The second aim of this study was to examine the concentration, morphology and motility of the resultant sperm populations following semen preparation using the PureSperm<sup>®</sup> density gradient and swim-up techniques. Semen preparation is an essential step in any fertility treatment protocol, and it is important that the sperm obtained following semen preparation has sperm morphology and motility characteristics capable of improving assisted fertility success rates. Currently, the PureSperm<sup>®</sup> density gradient and sperm swim-up are the most widely employed techniques in fertility clinics. Although there is sufficient evidence to suggest they are each effective at extracting sperm with improved quality from neat semen, there remains insufficient evidence to suggest which of these two techniques is superior. The present investigation revealed that both sperm preparation methods were effective

at improving sperm morphology and motility, however to varying degrees. The swim-up method yielded a population of sperm with superior motility and morphology when assessed according to World Health Organisation (WHO) criteria, while the PureSperm<sup>®</sup> density gradient technique isolated a higher percentage of normal sperm, according to both WHO and Tygerberg strict criteria, with motility better than that of neat semen. Although results obtained via the swim-up method suggest it would be best for use in *in vitro* fertilization (IVF), the very low concentration of sperm isolated via this method remains a significant draw-back. The PureSperm<sup>®</sup> density gradient separation technique on the other hand is capable of isolating larger quantities of sperm, which is likely to be of more benefit with fertility treatments requiring larger quantities of sperm. Based on these findings, the use of PureSperm<sup>®</sup> density gradient technique is recommended, due to its ability to isolate large quantities of good quality sperm. However, a swim-up may still be of use when performing fertility treatment using a sperm sample which possesses a high concentration and motility.

## OPSOMMING

Sperm morfologie bly 'n belangrike parameter in die voorspelling van vrugbaarheid, beide in vivo en in vitro. Tog is daar nogsteeds 'n aansienlike vlak van kommer rondom die ware potensiaal van hierdie parameter weens die gebrek aan standardisering van verskillende kleuringstegnieke wat gebruik word vir die evaluering van spermmorfologie. Hierdie studie is daarop gemik om ondersoek in te stel na twee algemeen gebruikte kleurings tegnieke naamlik, Rapidiff<sup>®</sup> (RD) en Papanicolaou (PAP), asook 'n nuwe kommersiële beskikbare kleurstof, SpermBlue<sup>®</sup> (SB), vir die evaluering van spermmorfometrie en morfologie. Resultate dui aan dat beduidende verskille in sperm morfometriese afmetings ontstaan as gevolg van die gebruik van die verskillende kleurstowwe. Bevindinge dui verder daarop dat RD swelling van die sperm se kop versoorsoak, terwyl PAP die spermkop laat krimp. Resultate wat verkry is met behulp van die SB kleuringstegniek dui daarop dat hierdie kleurstof aanleiding gegee het tot afmetings naaste aan die verkry tydens die beoordeling van vars, ongekleurde sperme. Die gebrek aan standardisasie en die uiteenlopende effekte wat verskillende kleurstowwe het op die spermstruktuur en die evaluering van sperm morfologie ingeheel is kommerwekkend, veral tydens die evaluering van pasiënte met grensgeval morfologie. Op grond van hierdie resultate, word die gebruik van die SB kleuringstegniek, bo die gebruik van RD en PAP, vir effektiewe en akkurate morfologie evaluering aanbeveel. Verdere ondersteuning vir die gebruik van die SB kleuringstegniek is die feit dat daar bevind is dat SB 'n vinnige en eenvoudige metode is, wat toelaat vir maklike visualisering van sperme deur rekenaargesteunde sperm analise sisteme soos die Sperm Class Analyzer (SCA<sup>®</sup>).

Die tweede doel van hierdie studie was om die konsentrasie, morfologie en die motiliteit van spermopulasies te ondersoek, soos verkry tydens die voorbereiding van semen deur gebruik te maak van die PureSperm<sup>®</sup> digtheidsgradiënt en op-swem tegnieke. Die voorbereiding van semen is 'n noodsaaklike stap in enige vrugbaarheidsbehandeling protokol, aangesien dit belangrik is dat die sperme wat deur hierdie prosesse verkry word oor die nodige morfologiese en motiliteit eienskappe beskik wat in staat is om die sukses van vrugbaarheidsbehandelings te verbeter. Huidiglik is die PureSperm<sup>®</sup> digtheidsgradiënt en op-swem tegnieke die

mees algemeen gebruikte tegnieke in vrugbaarheidsklinieke. Alhoewel daar voldoende bewyse is wat voorstel dat elke tegniek effektief is vir die ekstraksie van sperme met beter kwaliteit vanuit semen, bly daar steeds onvoldoende bewyse wat daarop dui dat een van hierdie twee tegnieke beter is as die ander een. Huidige navorsing het getoon dat beide sperm voorbereidings metodes daarin geslaag het om sperme met normale morfologie en beter motiliteit te selekteer. Die opswem metode het 'n spermpopulasie met beter motiliteit en verbeterde morfologie gelewer, soos getoets volgens die WGO kriteria, terwyl die PureSperm digtheidsgradiënt tegniek sperme met verbeterde morfologie, volgens beide die WGO en Tygerberg Streng Kriteria, en 'n redelike verbetering in sommige motiliteits parameters geselekteer het. Hoewel die resultate wat verkry word via die op-swem metode voorstel dat dit die beste metode vir die gebruik tydens in vitro bevrugting sou wees, bly die baie lae konsentrasie van sperme wat met hierdie metode verkry word 'n belangrike nadeel. Die PureSperm<sup>®</sup> skeidingstegniek laat egter toe vir die isolering van groter hoeveelhede sperme, wat waarskynlik meer voordelig sal wees vir bevrugtingsbehandelings wat meer sperme benodig. Gebaseer op hierdie bevindinge, word die gebruik van die PureSperm<sup>®</sup> digtheidsgradiënt tegniek aanbeveel, as gevolg van hierdie tegniek se vermoë om groot hoeveelhede goeie gehalte sperm te isoleer. Daar kan egter nogsteeds van op-swem metodes gebruik gemaak word tydens vrugbaarheidsbehandeling indien die semenmonster beskik oor 'n hoë konsentrasie sperme met goeie beweeglikheid.

## **DEDICATION**

This dissertation is dedicated to my parents,

Graham and Gaye McAlister.

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## ALPHABETICAL LIST OF ABBREVIATIONS

ALH:	Amplitude of Lateral Head Displacement
ART:	Assisted Reproductive Technologies
BCF:	Beat/Cross Frequency
BSA:	Bovine Serum Albumin
CASA:	Computer Aided Semen Analysis/Analyzer
GIFT:	Gamete Intra-fallopian Transfer
ICSI:	Intracellular Sperm Injection
IMSI:	Intracytoplasmic Morphologically Selected Sperm Injection
IUI:	Intra-uterine Insemination
IVF:	In Vitro Fertilization
PAP:	Papanicolaou
RD:	Rapidiff <sup>®</sup>
ROS:	Reactive Oxygen Species
SB:	SpermBlue <sup>®</sup>
SCA <sup>®</sup> :	Sperm Class Analyzer <sup>®</sup>
VAP:	Average Path Velocity
VCL:	Curvilinear Velocity
VSL:	Straight Line Velocity
WHO:	World Health Organization

WOB: Wobble

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# CHAPTER 1

## INTRODUCTION AND AIM OF STUDY

### 1.1 Introduction

Routine semen examination remains an important tool in the diagnosis and treatment of human subfertility (54, 82). Although various factors are considered, concentration, motility and morphology of sperm are generally recognized as the three most important parameters to be assessed (47, 68, 79, 84). These parameters are considered most useful as they have been shown to indicate fertility potential, albeit to varying degrees (84, 97). For instance sperm concentration, which refers to the number of sperm present in one millilitre of semen, has been shown to correlate with fertility rates, where very low concentrations have been shown to deem a male subfertile (42). Consequently, sperm concentration is an important factor to consider during fertility treatment.

Spermatozoa, after passage through the epididymis, become motile. Motility is a particularly important function which enables the delivery of sperm to the site of fertilization in the female genital tract (105). Furthermore, this factor becomes critical at the time of fertilization since it facilitates passage of the sperm through the zona pellucid (25, 78). For these reasons, motility indicates sperm functional capacity, and is thus considered a valuable indicator of a man's fertilization potential (11, 42). *In vitro*, motility remains a particularly important parameter when couples are undergoing Intrauterine Insemination (IUI), Gamete Intra-fallopian Transfer (GIFT) and In Vitro Fertilization (IVF), as it has shown to be predictive of the success of the given fertility treatments (11, 31).

Of all semen parameters however, sperm morphology appears to be one of the most powerful indicators of a man's fertility potential both *in vivo* and *in vitro* (56, 82). A sperm cell is considered normal if it conforms to the criteria classifying normal morphology, including the size and shape of the head, neck and tail (43). Abnormal sperm morphology may be a marker of underlying pathology, such as impaired sperm

function or decreased DNA integrity, which may directly or indirectly result in impaired fertilization rates (78) or decreased embryo quality (92, 102).

Since the first successful IVF pregnancy in the early 1970's, the number of fertility treatment options has vastly expanded (103). An integral step in each treatment process involves preparing the gametes for use *in vitro*. As with the expansion of fertility treatment options, multiple methods for gamete preparation now exist (74). However, it is crucial that from the many existing semen preparation techniques, one is chosen which optimizes the aforementioned sperm parameters (sperm concentration, motility and normal morphology), thereby enhancing the potential for a successful pregnancy.

## **1.2 Objective and statement of the problem**

Although the importance of sperm morphology is acknowledged, with the lack of standardization relating to preparation, evaluation and staining techniques used in morphology assessment, the possibility exists that the true potential of this parameter has not yet been reached. However, with the availability and use of the computer aided semen analysis (CASA), the subjectivity of morphology analyses has been somewhat lessened (37). On the other hand, the lack of standardization surrounding the staining techniques used in the evaluation of sperm morphology may explain the discrepancies found in a number of comparative studies (45, 64). It has been suggested in previous publications that the use of different staining techniques could possibly influence the outcome of the number of morphologically normal sperm. Under such circumstances, a patient may be classified as having normal sperm morphology by one treatment centre and abnormal by another (37, 68). This may become particularly challenging for physicians comparing semen analyses among laboratories which use different techniques (54).

In addition to the variety of staining techniques used for morphology evaluation, a number of sperm separation methods are currently employed in fertility centres, in an attempt to isolate a subpopulation of sperm most likely to achieve fertilization of an oocyte (59, 74). Although a great deal of literature exists regarding the strengths and limitations of various semen preparation techniques, comparative studies yield

conflicting data, and there is insufficient evidence to recommend any particular technique for use during fertility treatment (21).

The aim of this study is therefore twofold:

- (i) to evaluate the differences of three different staining techniques (Papanicolaou, SpermBlue<sup>®</sup> and Rapidiff<sup>®</sup>) with regards to morphological and morphometric sperm evaluation, in order to identify which one has the least effect on sperm structure and gives the best indication of an unstained sample,
- (ii) to investigate the differences of two commonly used semen preparation techniques namely, the swim-up and PureSperm<sup>®</sup> density gradient methods, with regards to sperm yield, motility, morphometry and morphology evaluation.

Both topics under investigation in this thesis are particularly relevant to the field of subfertility diagnosis and treatment.

### **1.3 Plan of study**

Serving as a background to the study, an extensive overview of current literature regarding staining methods used for microscopic evaluation in fertility clinics, as well as different techniques used for the preparation of semen prior to fertility treatment, is provided in chapter 2. This is followed by the basic materials and methods in chapter 3. Chapters 4 and 5 comprise of the results and the discussion respectively.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Introduction

The first step in an subfertile couple's treatment process involves determining the cause of subfertility, both in the male and the female. For this reason a routine semen evaluation forms an integral tool in the diagnosis and treatment of male factor subfertility (37, 53, 82). Since the fertilizing ability of sperm involves numerous functional aspects such as motility and the acrosome reaction, impairments of these functions may individually cause fertilization failure both *in vivo* and *in vitro* (84). Therefore, in the assessment of male fertility, it is standard procedure to quantify various semen and sperm parameters. Although many factors which are likely to influence or at least indicate the potential for fertility are routinely assessed (including semen pH, viscosity, colour and odour) (67, 111), sperm concentration, motility and morphology are generally considered the three most important and informative parameters (68). These parameters have shown to be particularly useful in the diagnosis of fertility problems between couples, as well as in the prediction of ART success. Although the routine semen evaluation is valued by fertility clinicians worldwide, the reliability of the relevant tests are confounded by a lack of standardization regarding sample preparation and evaluation (53).

Sperm morphology evaluation, which has been shown to be one of the most reliable parameters in indicating a man's fertilizing ability (24, 78), involves the staining and visualization of a semen smear under a microscope, where it is graded by selected criteria. The lack of standardization is introduced when the methods of preparation and assessment vary between clinics, leading to a considerable variation in readings (27, 52, 54). The lack of standardization is especially problematic when treating subfertile couples who were referred from other clinics. Due to discrepancies between laboratories for example, a patient could very well be classified as normal by one laboratory and subfertile by another (53, 54). Though in recent years, the dilemma surrounding the subjectivity of morphology evaluation has been somewhat rectified

by the introduction of Computer Aided Semen Analysis (CASA) system, the main cause for concern lies with sample preparation and the various morphology staining techniques employed world-wide (59). As a result of the varying effects morphology stains have on the sperm cells, border-line forms may be differently analysed. Possibly, with the introduction of a standard staining procedure, the true potential of the morphology evaluation can be attained.

Following a complete semen evaluation, a subfertile couple may choose to commence with fertility treatment. As with any fertility treatment program, an essential step in the process involves the appropriate preparation of the male and female gametes *in vitro* (74). Currently, several semen preparation techniques exist, and may be employed for a variety of reasons, the main ones being to rid the sample of harmful factors and isolate the required sperm subpopulation (12, 76). Although numerous studies surrounding different preparation techniques have been done, there remains no consensus as to which method is more effective at isolating functionally superior sperm (86, 89). Provided a technique can be recommended, fertility clinics may benefit by saving both time and money, along with potentially increasing the fertility treatment success rates.

The importance of three particular semen parameters, the issues surrounding the lack of standardization in morphology evaluation, as well as a review of current literature regarding various semen preparations and the subpopulations they yield in relation to sperm concentration, motility and morphology will be discussed in the remainder of this chapter.

## **2.2 Routine semen analysis**

The goal of accurately estimating a man's fertility potential has long been of great interest to researchers and clinicians alike. It is however important to recognise that *male subfertility* is not a term defining a specific clinical syndrome but rather a collection of different conditions exhibiting a variety of aetiologies and varying prognoses (94). At present, approximately 15% of couples world-wide are unable to conceive a child within 1 year of regular unprotected intercourse and it has been estimated that a male factor is solely responsible in well over 30% of these cases (31, 105). A semen analysis is the most important source of information regarding the fertility status of the male partner, whereby it assesses the potential for fertility, rather than being a test for actual fertility. If a male subfertility factor is present, it is usually defined by abnormal parameter readings during a routine semen analysis (95).

### ***Specimen collection***

In order to accurately interpret a semen analysis, the clinician needs to know the method by which the sample was produced, the approximate time lapse between the production and analysis, as well as the days of abstinence and type of container used. These factors may have a pronounced influence on the results obtained through a semen evaluation. With the intention of standardizing the semen evaluation process, the World Health Organization (WHO) provided some guidelines for sample collection. These guidelines advise that the patient produces the sample on site or in close proximity to the laboratory, in an appropriately equipped room and by means of masturbation without lubrication. However, depending on the patient's wishes, other methods may be used. It is generally accepted that the period of abstinence has an effect on semen parameters, particularly volume and sperm concentration. It is therefore prescribed that prior to sample collection, the patient is to abstain from ejaculating for 2-7 days. This is primarily to standardize the conditions of evaluation and to reduce inter-sample variations. Once collected, the sample should be delivered to the laboratory within 30 minutes of ejaculation, preferably keeping it warm or as close to body temperature as possible. The sample analysis should begin within 30-40 minutes after ejaculation, during which time the semen should have liquefied, allowing for the free movement of the sperm (111).

### ***Evaluation of physical characteristics of semen***

A spermatozoon is a highly specialized haploid cell whose function can be influenced at various levels, directly and indirectly. The standard semen analysis includes the assessment of both physical and quantitative parameters. Physical characteristics may indicate underlying problems that might call for closer examination. One of the first steps in evaluating a semen sample is to characterise its colour and consistency (26). The average sample is a thick coagulum, milky-white in colour which liquefies about 30 minutes post ejaculation, becoming very watery and fluid-like (40, 111). Once liquefaction has occurred, the sperm are able to swim freely. Failure of liquefaction taking place may hinder sperm movement, ultimately affecting the fertilization process. An additional physical characteristic routinely assessed includes semen volume, which indicates the functioning of accessory reproductive glands such as the seminal vesicles and prostate gland. Furthermore, pH and odour are noted as these characteristics may be a sign of infection or accessory gland dysfunction (111) (*See Table I*).

### ***Evaluation of qualitative characteristics of sperm***

Following a physical macroscopic evaluation of the semen, the sample is then examined on a microscopic level with the intention of evaluating functional parameters such as sperm motility, viability, morphology and concentration, all of which signify fertility potential to varying extents (62). The presence of leukocytes, immature sperm cells, anti-sperm antibodies and bacteria are also routinely investigated (7), since these factors may suggest underlying abnormalities such as infection or disorders of spermatogenesis, both of which can adversely influence fertility. Of the aforementioned parameters however, sperm concentration, motility and morphology are considered to be the most important (68, 84, 93). These three parameters are known to be the most informative in the prognosis of subfertility, both *in vivo* and *in vitro*, and are thus the focal point in the majority of semen evaluations (68). Sperm concentration, motility and morphology will be discussed in greater detail in the following sections and form the foundation of the aforementioned research topic.



**Table I.** Normal values for semen parameters according to WHO 1999 guidelines

<b>Parameter</b>	<b>Reference values</b>
Volume	≥2.0 mL
pH	± 7.2
Sperm concentration	≥20 X 10 <sup>6</sup> spermatozoa/mL
Total sperm number	≥40 X 10 <sup>6</sup> spermatozoa per ejaculate or more
Motility	≥50% total motility or ≥25% progressive motility
Morphology	WHO: >30% normal Tygerberg strict criteria: >14% normal
Vitality	≥50% or more live, i.e. excluding dye

### ***Computer Aided Sperm Analysis (CASA)***

In the past, fertility clinics frequently had to contend with the unreliability and inaccuracy of manual sperm morphology evaluations, thereby reducing the confidence in the outcome and predictive value of the standard semen analysis (22, 23, 47). In studies where manually evaluated sperm morphology outcomes were compared, it was evident that observer bias resulted in discrepancies between the results, owing to the subjective nature of the evaluation process (22, 85). Since then, the extreme level of inter- and intra-laboratory variation in manual sperm morphology evaluation practised world-wide, has been repeatedly illustrated (22, 30). Consequently, this lack of precision surrounding manual visual assessments led researchers and clinicians to question the overall clinical value of the semen evaluation. These shortcomings soon resulted in the development of CASA, which promotes standardization by being a more objective and precise tool for semen evaluation (22). Recent studies have confirmed this by showing that employing two different CASA systems yields a high level of precision and reliability (22, 23, 97).

CASA systems are used for assessing sperm viability, DNA fragmentation, motility, concentration and morphology. These systems are advantageous over manual methods as they are capable of providing additional information that would not be attained through manual assessments. For instance, in the case of sperm motility assessment, CASA is able to provide additional quantitative data on sperm kinematic parameters (23, 37). These particular parameters may provide valuable information relative to the quality of the sperm motion, which in recent years has become increasingly relevant in the assessment and prediction of fertility (11, 78). In addition to the advantages the CASA may provide in a clinical setting, sperm kinematic parameters may be particularly useful in the research setting to allow for a better understanding of sperm function.

### **2.2.1 Sperm Concentration**

#### ***Biological importance***

Sperm concentration or the number (expressed in millions) of sperm per millilitre of seminal fluid, is an accurate measure of spermatogenesis and therefore one of the most critical determinants of male subfertility, as defined by the WHO (16, 97). Where the human female releases on average only one oocyte per month, males differ greatly by producing and releasing millions of sperm in a single ejaculate. The female reproductive tract is an environment of several hazards, where immune responses, low pH, cervical mucus and simply the length of the passage can be detrimental to sperm survival (77, 99). Such obstacles might represent physiological filters for sperm with imperfect genetic material, so that in some sense it is the fittest which survive. Thus, the excessive number of sperm released in an ejaculate can be seen as reflecting the heavy odds against survival.

#### ***Clinical importance***

The importance of sperm concentration can be confirmed, as it has been shown repeatedly that in comparison to men with a normal sperm count, men with subnormal concentration have a reduced fertility rate *in vivo* (10, 42). According to the WHO, a semen sample is normal if the concentration is  $\geq 20$  million/mL, or at least possesses a

total sperm count of 40 million in the entire volume of the ejaculate (111). Hence, a man with a sperm concentration of less than 20 million/mL is considered subfertile, and will more than likely encounter fertility problems *in vivo*.

It is well known that sperm concentration is important in natural fertilization, though presently with the refinement and expansion of artificial reproductive procedures, this semen parameter may play less of an important role *in vitro* (16). It has been suggested in a study by Byrd et al. (1987), that in ARTs such as IVF, ICSI and IUI the required sperm concentration might be much lower than 20 million/mL. Considering these techniques, it is suggested that only one sperm is needed for ICSI, about 50 000 for IVF, and 1 million or fewer motile sperm for IUI, (15, 16). In spite of this, sperm concentration still plays an important role in determining which method of fertility treatment would be most suitable for the couple. Therefore, despite the introduction of ICSI where only a single sperm is required for use *in vitro*, concentration is still a factor to consider when fertility treatments such as IVF or IUI are to be performed with patients displaying severe oligozoospermia (107). In such cases, where cheaper alternatives to ICSI are to be attempted first, the appropriate semen preparation technique which suitably prepares the semen without further decreasing the sperm concentration should be considered.

### **2.2.2 Sperm motility**

#### ***Biological importance***

At the time of ejaculation, when mixed with the secretions of the accessory sex glands, sperm become motile cells (13, 72). Sperm motility is generated by a long, whip-like tail composed of propulsive flagella, energy for which is provided by the mitochondrion-dense mid-piece (36). Where sperm count is an accurate measure of the effectiveness of spermatogenesis, motility is a measure of epididymal maturation and sperm functional capability (16). Therefore, the quantity of motile sperm in an ejaculate is possibly more important than sperm concentration or sperm count alone. Cases where the concentration presents as normal, is not of much value when the sperm are immotile and non-functional, as motility is crucial for successful fertilization which demands migration of the sperm through the harsh environment of

the cervix to the ovum (73). Not only is motility required for transportation, but flagellar activity is also vital at the site of fertilization where motility is the mechanical driving force behind the penetration of the sperm through the outer layers of the ovum (78). For these reasons, assessment of sperm motility can provide important information of sperm function and fertilization capability.

### ***Clinical importance***

For the assessment of sperm motility, a simple grading system is recommended that distinguishes progressive and non-progressive motility from immotile spermatozoa. Motility assessment involves grading each sperm as being type a, b, c, or d according to the particular motility characteristics it displays. *Type a* sperm display rapid, progressive motility, and swim at a speed of 25  $\mu\text{m/s}$  or more at 37°C, which is approximately equal to the movement of 5 head lengths or half a tail lengths distance in one second. *Type b* display progressively motile sperm, swimming in a forward fashion, but slower and more sluggish than *type a* sperm. Non-progressive sperm are classed as *type c*, where the sperm is motile, however does not display forward progression, but rather an irregular swimming pattern at less than 5  $\mu\text{m/s}$ . Lastly, those sperm displaying a total absence of motility, are deemed immotile and categorised as *type d* sperm (111).

Clinicians are particularly interested in the progressive motility or the total concentration of *type a* and *b* sperm, as this best indicates the ability of the sperm to move in a forward fashion towards an oocyte (78). According to WHO guidelines, sperm motility is normal when 50% or more sperm are progressively motile (*type a* + *b*) or 25% or more are rapidly motile (*type a*) at one hour after ejaculation (62, 78). Progressive motility has been shown on numerous occasions to be a useful parameter in the prediction of fertility success both *in vitro* and *in vivo* (32, 104). For instance, it has been repeatedly demonstrated that motility is a particularly useful parameter in the prediction of IVF (84), GIFT and IUI success (11). For example, in a study by Miller et al. (2002), it was shown that processed total motile sperm count independently predicts success with IUI, where cycles with less than 10 million total motile sperm are significantly less likely to result in a pregnancy (70). Similar findings were reported in other investigations regarding IVF success (70, 107).

During a process known as capacitation, sperm undergo two important physiological changes, namely hyperactivation and the acrosome reaction (29, 61) (*See Section 2.2.3 for more on the acrosome reaction*). Capacitation is induced by numerous factors such as sterol binding albumin, lipoproteins, proteolytic and glycosidase enzymes, all naturally found in the female reproductive tract (39, 41). Capacitation involves the destabilization of the acrosomal sperm head membrane, rendering it more fusogenic, with an increased permeability to  $\text{Ca}^{2+}$ . An sudden influx of  $\text{Ca}^{2+}$  leads to elevated intracellular cAMP levels which in turn causes an increase in motility (38, 61).

This important type of movement displayed by capacitated sperm is specifically known as hyperactivated motility and is characterised by sharply curved flagellar beats and a circular or erratic swimming trajectory (100). Consequently, hyperactivation and its distinctive asymmetrical path is used as a visual indication that a sperm cell has undergone capacitation. Several biological functions have been proposed for hyperactivation. These include increasing flexibility for moving sperm out of pockets created by mucosal folds, disengaging sperm from adherence to oviductal epithelium and increasing the chance a sperm cell will encounter the egg in the oviductal lumen. Other functions of hyperactivity include facilitating the penetration of sperm through viscous and viscoelastic substances such as oviductal mucus and the cumulus matrix and more importantly, facilitating the penetration of sperm through the zona pellucida during fertilization (96, 100). Several commonly used components are essential for successful *in vitro* capacitation of sperm. Among them are bovine serum albumin (BSA),  $\text{Ca}^{2+}$  and bicarbonate ( $\text{HCO}_3^-$ ). (39, 98, 112).

In recent years with the introduction of the CASA system, the task of measuring sperm motility parameters has become much easier. Computerized motion parameters or motility kinematics, which describe the movement of sperm in time and space (75) have been reported to be predictive of ART results (3, 84, 88). Three velocity parameters are measured by CASA, namely the straight-line velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP). From these measurements progression ratios can be calculated, giving linearity (LIN), straightness of the average path (STR) and wobble (WOB) of the sperm head about the average path. Furthermore, amplitude of lateral head displacement (ALH) and

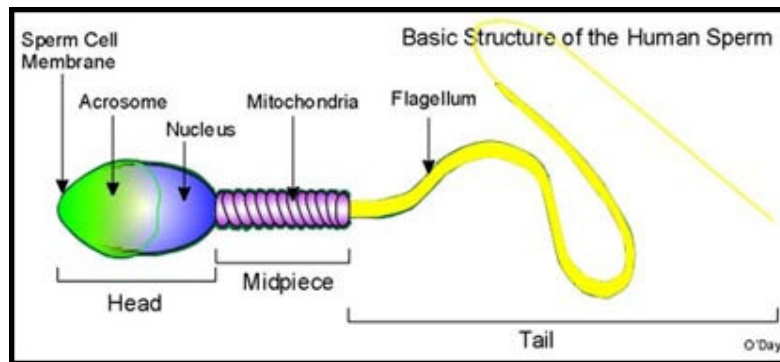
beat-cross frequency (BCF) are measured. ALH is calculated from the amplitude of the lateral head deviations of sperm head about the axis of progression, whereas BCF signifies the number of times the curvilinear track crosses the average path per unit of time, which also indicates the flagellar beat frequency and frequency of rotation of the head. Together, motility kinetic parameters enable a greater understanding of the patterns and characteristics of sperm motility. Consequently, a large amount of evidence suggests that some CASA velocity parameters provide a reliable estimation of the fertilizing ability of human sperm (48). To support this, an early study by Holt et al. (1985) showed a direct correlation between VCL of sperm and IVF results. Since then, similar findings have also established a strong relationship between this particular velocity parameter and the success of fertility treatment (3, 48, 88). Additionally, relationships between ALH, LIN, VSL (3, 11, 31, 78, 88) and VAP with IVF results have since been established (16, 78). These correlations with IVF may provide useful information for the management of patients requiring fertility treatment.

### **2.2.3 Sperm morphometry and morphology**

#### ***Biological importance***

Although mammalian sperm are characteristically small, they are known to vary considerably in size and shape (36, 37). In earlier years, it was discovered by microscopic examination of sperm in an ejaculate that the overall morphology is noticeably heterogeneous, with a single ejaculate containing sperm of many different shapes, sizes and forms (37). This prompted scientists to identify and define the morphological characteristics of a normal sperm. Observations of sperm recovered from the female reproductive tract, especially in post coital mucus, or from the surface of the zona pellucida, were found to have a homozygous appearance and have helped define a normal sperm (111). During migration through the cervical mucus, a strong selection for certain morphological types of sperm occurs. This positive selection results in a population of spermatozoa with a significantly increased morphological uniformity compared with the population in the original semen (30).

A sperm is morphologically divided into three main parts (*See Figure 1*), namely the head, midpiece and tail region (79). A normal head, containing the sperm's complement of genetic information, has a smooth oval configuration and is 4.0-5.0 $\mu\text{m}$  in length and 2.5-3.5 $\mu\text{m}$  in width according to the WHO (*See Table II*) (111). The sperm head is capped by an acrosome, which should occupy 40-70% of the total head area. The acrosome serves a vital function during fertilization, as it contains enzymes necessary for the penetration of the oocyte. The midpiece, containing a number of mitochondria necessary for the provision of energy for sperm movement (79), should be uniform,



*Figure 1. An illustration showing the basic structure of a human sperm (80)*

slender, approximately 1 $\mu\text{m}$  thick and about one and a half times the length of the head. Furthermore, a normal tail is defined as one that is straight, uniform and thinner than the midpiece, and is approximately 45 $\mu\text{m}$  long (111). Sperm which do not confine to the given criteria are considered morphologically abnormal, and it is possible that a single sperm possess more than one abnormality.

**Table II.** Normal ranges for sperm morphometry according to WHO guidelines

<b>Morphometric parameter</b>	<b>Reference value</b>
Head length	4.0 – 5.0µm
Head width	2.5 – 3.5µm
Acrosome coverage	40 – 70% of head area
Midpiece length	6 – 10µm
Midpiece width	± 1.0µm
Tail length	± 45µm

### ***Clinical Importance***

Of all semen parameters, sperm morphology is probably one of the best indicators of a man's fertility potential, as it has been shown to be the most stable parameter and has the advantage of being predictive of fertility success (37, 79). For this reason, sperm morphology and its relation to fertilization ability *in vivo* and *in vitro* has been studied intensively. Studies have suggested that sperm morphology assessment by relatively simple and inexpensive methods can provide prognostic information similar to that obtained from some of the more elaborate sperm function tests (14).

Two main classification systems for sperm morphology analysis currently exist, namely the WHO criteria and Tygerberg strict criteria (22). In contrast to WHO criteria, Tygerberg strict criteria, as the name suggests, is a more stringent method of analysis by which borderline forms are considered abnormal (22, 57, 67). Tygerberg strict criteria is based on the morphology of postcoital sperm found in good cervical mucus obtained from the endocervix (67). WHO criteria suggests that teratozoospermia is present only when the percentage of normal forms is less than 30%, whereas this value is lowered to 14% when applying Tygerberg strict criteria (22, 108). This threshold was obtained after noting that patients undergoing IVF with fewer than 14% normal forms had a significantly decreased fertilization rate than those with more than 14% normal forms (2, 19). According to Tygerberg strict criteria a total of 14% or more normal forms is regarded as a normal-pattern or n-pattern. The group possessing abnormal morphology according to Tygerberg strict criteria can be



further subdivided, which states that 4% or less normal forms be classified as a poor- or p-pattern, and 5 to 14% normal forms be classified as good- or g-pattern morphology (19). These groups further predict the possibility of obtaining a pregnancy with IVF treatment. G-group individuals have been shown to display a fertilizing ability that is lower than normal, although fertilization is still possible with IVF. P-group individuals, on the other hand, have been shown to have very low success rates with IVF (79).

These two classification systems, WHO criteria and Tygerberg strict criteria, are often used in comparative studies to establish superiority with regards to clinical prognostic value (22). On numerous occasions stricter criteria for normal morphology have been shown to be useful in the prediction of IVF success (30, 34).

### ***Sperm morphology as biomarkers for defective sperm***

Sperm morphology as assessed by strict criteria is recognized as an excellent biomarker of sperm dysfunction, determining the source of male subfertility and in predicting the outcome of assisted reproductive technologies (30, 85, 102). Numerous studies have shown that sperm morphology is significantly different in fertile when compared to subfertile men (62, 85, 102), where there is a definite positive correlation between the percentage of morphologically normal sperm and fertility (37, 109). Consequently, sperm morphological abnormalities can be indentified in a large proportion of patients with failed fertilization (109), particularly when assessed in accordance to strict criteria (59, 81, 102). Several reports have also verified that in patients with severe teratozoospermia, implantation rates are impaired, thus reducing the chances to establish a normal pregnancy (47, 62). Excessive sperm abnormalities may result from factors such as infections, drug use and fever, and as a consequence sperm morphology can often be used as an indicator of biological and toxicological stress (37, 109).

If a sperm cell is morphologically abnormal, it is likely not to possess the adequate machinery to progressively travel towards and fertilize an oocyte (102). In support of this, it has been reported that morphologically normal sperm swim faster and straighter (30, 84) where abnormally shaped sperm are generally less motile, and are

less successful at travelling through the female reproductive tract to the site of fertilization (30, 109). To maintain this, a number of independent studies have reported a high positive correlation between percentages of normal forms and progressive motility in whole semen (30, 63, 84). Some reports have also introduced the concept that to some degree, the zona pellucida is able to select morphologically normal sperm over abnormal sperm (30).

Morphological evaluation may also indicate to a certain degree, the functional capacity of the sperm with regards to acrosome function. The sperm's ability to capacitate is of vital importance in the fertilization process, whereby the acrosome releases hydrolytic enzymes and assists the sperm through the outer layers of the ovum (79). Failure to properly do so prevents natural fertilization from occurring. Semen containing sperm with low percentages of normal acrosomes is known to be associated with failed fertilization (18, 66) and morphology evaluation has been suggested to indicate to some degree the capability of a sperm cell to undergo an acrosome reaction. One study identified a close correlation between sperm head defects and decreased responses to acrosome reaction inducers (84). By simple morphology evaluation of the acrosome, clinicians can predict to some degree the physiological capability of the sperm to capacitate (66).

In addition to the correlation established between morphology and particular sperm functions, it has been previously suggested that sperm head abnormalities may be markers for other defects that significantly impair fertility, for instance genetic aberrations (58, 79, 102). To maintain this, a number of investigations have found a strong positive relationship between sperm head defects and DNA abnormalities. A particular study by Zini et al. (2009), compared sperm head abnormalities with DNA integrity, and found a significantly higher level of genetic disturbances in teratozoospermic patients, suggesting that sperm head defects may in part be due to reduced nuclear compaction. As a consequence of reduced chromatin condensation, it was suggested that there may be far less protection against external stressors, which predisposes the DNA to oxidative stress and harmful temperature fluctuations, ultimately leading to fertilization failure and subfertility (115).

Along with fertilization, it has been suggested that sperm is involved in the embryonic quality and the early stages of development. This theory has been motivated by demonstrating an association between abnormal sperm morphology and poor embryo morphology (62). Although the importance of sperm morphology is well and truly established in an IVF scenario, clinicians were uncertain of the role it would play in fertility prediction in the new era of ICSI. It was subsequently discovered in cases where ICSI was performed with sperm from teratozoospermic men, that although fertility and cleavage rates were acceptable, a high incidence of failed implantation and early pregnancy loss were encountered (102). This finding strengthened the assumption that abnormal sperm morphology is not only important for the migration of the sperm to the oocyte and at the site of fertilization, but also in the quality of the sperm and DNA necessary to sustain a pregnancy.

It however must be stated that as long as there is a morphologically normal sperm available for injection, it seems that the outcome of ICSI is not related to the incidence of morphologically abnormal spermatozoa in the sample. In support of this, a study showed that the conception rates following the use of the most advanced technique of assisted reproduction (ICSI), were shown to be independent of the number of morphologically abnormal spermatozoa (97). Although implantation and ongoing pregnancy rates may be lowered, ICSI seems to be one of the few treatment options in cases displaying total morphologically abnormal spermatozoa (102). However, a novel technique being introduced into the field of artificial reproduction namely, intracytoplasmic morphologically selected sperm injection (IMSI), may further increase the fertility success rates with teratozoospermic specimens. IMSI is a derivative of the standard ICSI technique, where more attention is paid to the quality of the sperm selected to be injected into the oocyte. Using this technique, a man's sperm is examined under a high-definition microscope, and only those sperm which appear to have morphologically normal nuclei are selected for fertilization of the partner's oocytes (9).

On the whole, sperm morphology may give the clinician an reasonable understanding of the functional capabilities and quality of sperm, which in turn indicates the chances of successful fertilization (45) and pregnancy. Therefore, during the assessment of sperm morphology it is important to select a staining technique which will most

accurately indicate a man's fertility potential. In addition to this, a semen preparation technique which isolates and optimizes the number of normal sperm is essential prior to fertility treatment.

## **2.3 Sperm morphology staining techniques**

The true potential of sperm morphology evaluation as a predictor of male fertility has been confounded by a multitude of factors arising from considerable variations in visual evaluation, sample preparation and staining techniques (24, 37, 54). The lack of standardization surrounding morphology evaluation has led many to question the reliability of this semen parameter (24). In recent years with the development of various CASA systems, subjectivity of the morphology evaluation has been addressed to a large degree, leading to more objective analyses. However, there remains a level of uncertainty surrounding different staining techniques, their effect on borderline forms and the resultant number of morphologically normal sperm encountered during an analysis (85). World-wide there is no specific recommended staining technique, although currently, the WHO suggests the use of the Papanicolaou (PAP), Shorr and DiffQuik stains (111). Consequently, two of the most widely-used staining techniques for the evaluation of sperm morphology include PAP and DiffQuik. Recently, a new stain, namely SpermBlue<sup>®</sup>, has been introduced to the market. This new staining technique, although suggested for use in sperm morphology evaluation, has not yet been substantially investigated.

### **2.3.1 Papanicolaou (PAP) staining technique**

The PAP staining method is possibly the most established and widely employed staining technique in andrology laboratories and fertility clinics. This multichromatic stain is considered a very reliable technique which involves the use of five dyes in three solutions. On a well prepared specimen, it allows for the identification of the acrosome and post-acrosomal region of the sperm head, cytoplasmic droplets, midpiece, and tail (111). Nuclei are stained blue while cytoplasm displays varying shades of blue, orange, pink or red. Although this staining method allows for suitable visualization of sperm, it is a very time-consuming process (56), involving multiple steps and solutions, for which reason it is being abandoned in favour of more rapid techniques. An additional drawback of the Papanicolaou staining method is that it is a relatively costly technique (55).

### **2.3.2 Rapidiff®(RD) staining technique**

Rapidiff®, a trademark name of DiffQuik, is a rapid staining technique. The RD protocol is significantly faster than the traditional PAP staining technique, and has a staining-to-reading time of less than 7 minutes (45, 56). This staining procedure was introduced by Kruger et al. in 1987, when it was found to be comparable with the results of the PAP staining method (45). It is a concern however, that some smears stained using rapid procedures such as RD may cause a considerable amount of background staining, and may not always result in the same quality as the PAP stain.

### **2.3.3 SpermBlue®(SB) staining technique**

Recently a new rapid staining technique namely SB, has been introduced to the market by Microptic, S.L., Barcelona, Spain. It is a relatively fast and simple 2-step staining procedure, claiming equal or better results than that of PAP. The stain was developed to differentially stain all the components of the sperm including the acrosome, head, midpiece and tail in varying intensities of blue (64). The sperm head and acrosome stain light and dark blue respectively. The midpiece stains distinctly dark blue whilst the tail is stained a slightly lighter blue. SB is advertised as being equally suitable for unprocessed semen as it is for sperm processed using the swim-up method, Percoll™ and PureSperm® gradient preparations, using most culture media. However, at this stage SB has not been properly investigated and the scope and capabilities of this technique have not been entirely established. Although a study by van der Horst et al. (2009), has suggested that the SB staining technique be favoured over the traditional PAP technique and rapid staining methods.

### **2.3.4 Current literature surrounding sperm staining techniques**

Although some studies claim that alternative staining techniques are as effective and reliable as one another, other studies have shown marked differences between stains with regards to stain intensity, differentiation and contrast, but more importantly sperm size and shape, all of which may significantly influence the outcomes of morphology evaluation (22). These slight discrepancies in staining characteristics may become particularly problematic when evaluating a subfertile couple for possible

treatment options, especially with a patient whose morphology values fluctuate between the p-pattern and g-pattern groups (56).

The lack of consensus surrounding the use of different morphological staining techniques becomes evident in light of current literature surrounding differential staining for sperm morphology evaluation. Two independent studies comparing PAP and DiffQuik stains found no significant morphological differences between the two staining methods (56), suggesting that each stain will be equally effective and comparable to the other. On the other hand, another study reported inconsistencies in morphology evaluations of DiffQuik when compared to PAP (45). Furthermore, a number of investigations have shown that the DiffQuik method results in significant sperm swelling and background staining (2, 64, 111). Despite these findings, DiffQuik is still recognised by the WHO as an appropriate staining technique for human sperm morphology assessment (106). Literature suggests that the effect of various staining solutions on sperm size and shape are rarely taken into account and seldom acknowledged.

Variations in morphology readings due to the use of different staining techniques have led some clinicians to suggest that the choice of staining method depend on the purpose of the investigation (45). In one study, the suggestion was made that for routine purposes the PAP staining method be used, whereas DiffQuik should be used in the case where a quick indication of a patient's sperm morphology is required (45). Despite this recommendation, there is still some level of concern surrounding the influence of a particular staining techniques on morphometry values. An additional concern surrounding morphology evaluation is the time required for sample preparation. With the PAP stain, a large amount of time is required for the staining process which delays both the time until morphology evaluation and the commencement of clinical proceedings. What is ultimately required is a stain which has the ability to give the clinician or researcher the best indication of the true morphology status of a semen sample. Furthermore, only one standard method should be recommended for the preparation of morphology slides in order to ensure inter-laboratory comparability of results and to enhance the value of sperm morphology analysis for predicting fertility (68).

## 2.4 Sperm separation techniques

The human ejaculate is a combination of non-reproductive cells, motile, immotile, mature, immature and dead sperm as well as different types of seminal components such as debris, prostaglandins, and microorganisms (4, 5, 74). Dead sperm, white blood cells and bacteria, all of which may also be found in semen, are known to produce free radicals. Excessive quantities of free radicals may result in oxidative stress, which has the potential to damage the sperm and impair fertilization of the ovum (5, 12). It has been reported that prolonged exposure to seminal plasma after ejaculation can permanently diminish the fertilizing capacity of human sperm *in vitro* and contamination of prepared sperm populations with only traces of seminal plasma can diminish, or even totally inhibit, their fertilizing capacity (74). Under *in vivo* conditions, sperm with potentially functional parameters are separated from semen by active migration through the cervical mucus following coitus (77, 86, 99). Therefore, when the cervical barrier is bypassed during fertility treatment, a population of viable, motile sperm free from seminal plasma and debris is required (16, 86). For this reason, semen preparation is routinely performed before any fertility treatment (114).

It is essential that sperm are separated from the seminal plasma environment not only as soon as possible after ejaculation, but also as effectively as possible (12, 17, 42, 74). Apart from removing the sperm from a potentially harmful environment, separation techniques are employed to separate sperm with a normal appearance and adequate motility from the rest of the sperm in an ejaculate (12). This will enhance the chances of successful fertilization, whereby a better quality of sperm can be isolated and used for fertility treatment.

Since the introduction of the first successful IVF technique in 1978, a wide range of semen preparation methods have been developed (5, 46, 68). Starting from the simple washing of spermatozoa, separation techniques based on different principles like migration, filtration or density gradient centrifugation evolved (17, 46, 93). All of these techniques are capable of separating sperm from the seminal plasma, albeit to varying degrees. Sperm recovery rates, motility, morphology and degree of DNA damage are known to vary greatly between procedures (4). An ideal sperm preparation technique should be one which is cost-effective, involves the removal of



seminal plasma gently, efficiently and quickly, while at the same time isolating a high quality of sperm (5, 114). Currently, two of the most commonly used techniques in fertility laboratories include the swim-up and density gradient centrifugation technique using PureSperm<sup>®</sup> solutions.

#### **2.4.1 Sperm swim-up technique**

The swim-up method has the advantage of being the most simple and cheapest sperm separation procedure (46, 76), and is possibly the closest to a natural selection process as would occur *in vivo* following coitus. Under *in vivo* conditions, potentially fertile spermatozoa are separated from immotile spermatozoa, debris and seminal plasma in the female genital tract by active migration through the cervical mucus (46, 76). Through this process, weaker and possibly abnormal sperm will make no progression at all or die along the way, whereas the superior and stronger sperm may reach the site of fertilization (74).

A swim-up may be performed using either a washed or unwashed semen sample. During this method, liquefied semen is either layered beneath a culture medium, or the culture medium is carefully placed on top of a washed sperm pellet. During a subsequent incubation period, ranging from 15 – 60 minutes depending on the application, the progressively motile spermatozoa migrate from the semen layer into the culture medium. The inclusion of this migration step is considered to be functionally equivalent to the process by which human sperm escape from the ejaculate and colonize the cervical mucus (74). During this process, not only are progressively motile sperm selected, but depending on the constituents of the culture media they may also undergo physiological changes such as capacitation, which is a fundamental prerequisite for the sperm's functional competence with regard to acrosome reaction and hyperactivated motility, both of which are essential at the site of fertilization (46).

Numerous studies have claimed that a high quality of sperm is obtained via the swim-up procedure, where there has been found to be a significant improvement in the percentage of motile, viable and morphologically normal spermatozoa than in original semen (30).

### **2.4.2 Density gradient centrifugation**

The typical methodology for the density gradient centrifugation involves the use of continuous or discontinuous gradients. With continuous gradients, there is a gradual increase in density of the media from the top of the gradient to the bottom, whereas the layers of discontinuous gradient show clear boundaries between each other (74). Semen is placed on top of the density media with the lower density and is then centrifuged for approximately 15-30 minutes, depending on the selected technique. During this time, highly motile sperm move actively in the direction of the sedimentation gradient and can therefore penetrate the boundary quicker than poorly motile or immotile sperm. The result is a pellet at the bottom enriched with highly motile sperm (46).

In the past, Percoll™ had been a standard technique for sperm separation using density gradients, as it was claimed to have the best efficiency in selecting motile sperm with good fertilization ability. However, late in 1996, serious concern was expressed about the polyvinylpyrrolidone component and endotoxin levels of Percoll™. The polyvinylpyrrolidone was later replaced by silica stabilized with covalently bound hydrophilic saline, such as used in PureSperm® density gradient (46, 88). Studies have suggested that the PureSperm® density gradient is as effective as Percoll™ for the recovery of good, progressively motile sperm for use in artificial reproduction (17, 20). For this reason, the PureSperm® density gradient is a widely used product for the separation of sperm using differential gradients.

### **2.4.3 Current literature surrounding sperm separation techniques**

A semen preparation method that yields a population of good quality sperm is one among various important factors in the process of subfertility treatment. Although several studies have been published on the effectiveness of different methods, there is insufficient evidence to recommend any specific sperm separation technique (12, 21, 89). Comparative studies on sperm preparation methods have essentially investigated outcomes such as recovery rates and conventional semen parameters. However, these findings have been contradictory and there is no consensus on which one method is superior at isolating functionally superior sperm (89).

A number of comparative studies claim that both the swim-up and PureSperm<sup>®</sup> density gradient methods are equally as effective as the other in isolating motile and morphologically normal sperm (89, 114). Other studies have suggested that the swim-up technique results in a better isolation of motile sperm than does the density gradient technique. In one particular study, the PureSperm<sup>®</sup> density gradient method did not produce satisfactory pregnancy rates, and was therefore abandoned in favour of the swim-up method during IUI and IVF treatments (88). In further support of the swim-up method, a study showed that this method produces a slight increase in the number of sperm with bigger acrosomes (30), suggesting better fertilization ability. It has been stated that an additional advantage of the swim-up method is that it has been found to isolate sperm with higher DNA integrities (89, 114). Recent papers have reported that the swim-up methods select for velocity, beat frequency and beat amplitude as well as for normal forms and motility (30). Furthermore, numerous studies have claimed that it is much easier to perform as compared to Puresperm<sup>®</sup> density gradient centrifugation (89).

Although sufficient evidence exists to suggest the sperm recovery rate is lower with the swim-up technique than with density gradient separation techniques, it is said to produce better suspensions with higher sperm velocity and greater proportions of sperm with intact acrosomes and normal morphology (88). On the contrary, outcomes of a number of comparative investigations have suggested that the PureSperm<sup>®</sup> density gradient technique results in populations of sperm with higher progressive motility and a far greater recovery rate than the swim-up technique (5, 113, 114). A particular study reported that differential gradient centrifugation is better than swim-up in selecting sperm with normal morphology according to strict criteria (86). Other reports claim that the swim-up method should be abandoned altogether in favour of this technique.

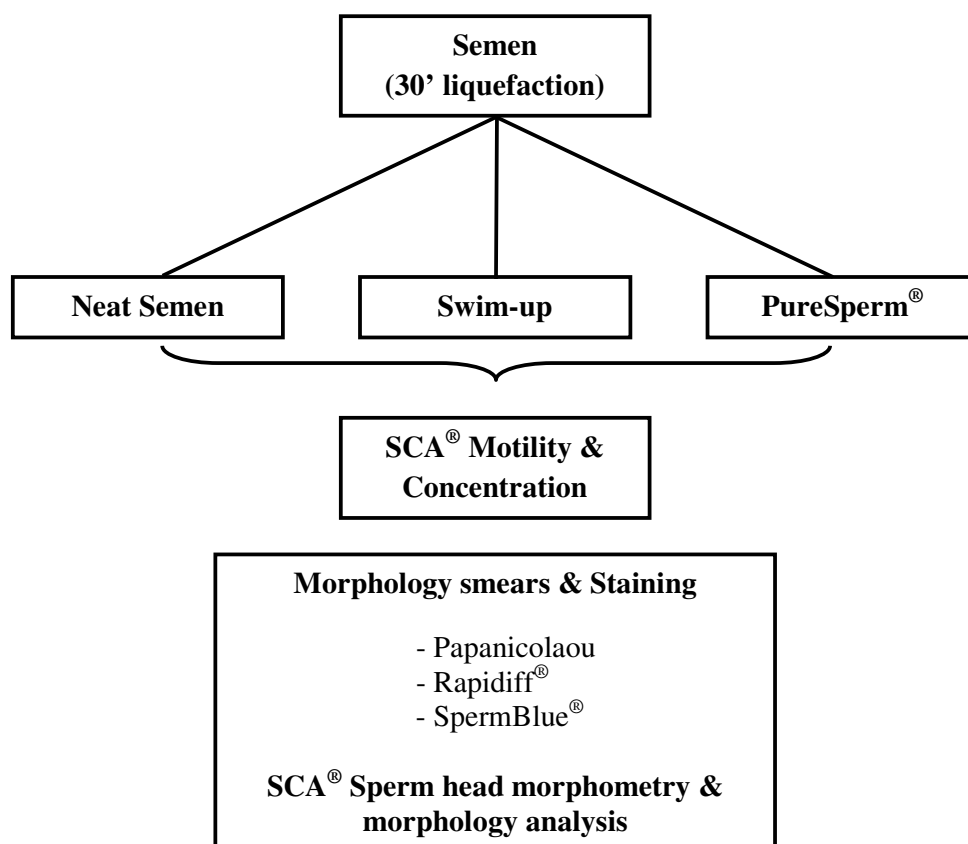
The vast amount of paradoxical evidence surrounding the effectiveness of these two techniques, explains the lack of consensus surrounding the best preparation technique for use in fertility clinics. By establishing a which of the two sperm separation techniques is best for clinical use, the success rates of fertility treatment may be optimised, while at the same time preventing unnecessary time and money expenditure.

# CHAPTER 3

## MATERIALS AND METHODS

### 3.1 Introduction

The detailed protocols and methods that were employed in this study will be outlined and discussed in this chapter. A brief outline of the experimental procedure that was followed is given in *Figure 2*.



*Figure 2: Flow chart showing the generalized experimental protocol*

The step-by-step outline of the experimental procedure depicted in *Figure 2* above is as follows:

- Step 1:** 30 minutes liquefaction @ 37°C
- Step 2:** SCA<sup>®</sup> Motility and concentration evaluation of neat semen
- Step 3:** Morphology smear & staining of neat semen (using three different staining techniques: Papanicolaou, Rapidiff<sup>®</sup> and SpermBlue<sup>®</sup>)
- Step 4:** Remainder of neat sample used for swim-up and PureSperm<sup>®</sup> 40/80 density gradient centrifugation sperm separation techniques
- Step 5:** SCA<sup>®</sup> Motility and concentration evaluation of sperm after separation
- Step 6:** Morphology smear & staining of sperm after separation (using three different staining techniques)
- Step 7:** SCA<sup>®</sup> Morphology analysis of stained smears (neat semen and sperm following separation) for all three staining techniques
- Step 8:** Tabulation of data, and statistical evaluation

### **3.2 Ethical Clearance**

Ethical clearance was obtained from The Health Research Ethics Committee.

ETHICS REFERENCE NUMBER: N09/09/232

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB): IRB0005239

### **3.3 Semen collection**

A total of 20 semen samples were obtained from healthy volunteer donors studying at the Tygerberg Campus, Stellenbosch University, aged between 19 – 24 years. A consent form was signed by each donor, ensuring them of their anonymity, that the sample was to be used for research purposes only, and to be disposed of accordingly following completion of experimentation. All semen samples were collected by means of masturbation after 2-3 days of sexual abstinence according to the WHO guidelines (111). Semen samples were collected in sterile wide mouthed containers. Shortly following collection, the semen samples were delivered to the laboratory where they were placed in an incubator at 37°C for 30 minutes and left to liquefy. Human sperm donor inclusion criteria was based on sample volume ( $\geq 2$  mL), sperm concentration ( $\geq 20 \times 10^6/\text{mL}$ ) and percentage of sperm motility ( $\geq 40\%$  total motility) (WHO, 1999). The final sample size was 20.

### **3.4 Preparation of Ham's-F10 culture medium**

Ham's Nutrient Mixture F10 is one of a number of media developed specifically to support the specific nutritional requirements and clonal growth of a variety of cells. HAMS-F10 has been shown to provide optimal nutrition and a safe environment for sperm *in vitro*.

The product was prepared according to manufacturer's guidelines which read as follows:

1. Measure out 90% of final required volume of water. Water temperature should be 15-20 °C.
2. While gently stirring the water, add the powdered medium. Stir until dissolved. Do not heat.
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.

4. To the solution in step 3, add 1.2 g sodium bicarbonate or 16.0 ml of sodium bicarbonate solution [7.5%w/v] for each liter of final volume of medium being prepared. Stir until dissolved.
5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
6. Add additional water to bring the solution to final volume. Ensure that the osmolarity is between 280 – 290 mOsm.
7. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns.
8. Aseptically dispense medium into sterile container.
9. Warm the solution to 37 °C before use.

### **3.5 Semen preparation**

Following the liquefaction period, the neat semen sample was analysed on the Sperm Class Analyzer<sup>®</sup> (SCA<sup>®</sup>) by Microptic, S.L., Barcelona, Spain, for concentration and motility, after which a small fraction was used to make a morphology smear. The remaining semen was then portioned and used for performing both a sperm swim-up and PureSperm<sup>®</sup> 40/80 density gradient centrifugation separation.

#### **3.5.1 Swim-up**

For the sperm swim-up technique, a specific volume of culture medium (Ham's F10) containing 3% bovine serum albumin (BSA) was transferred to a 15 mL Falcon tube. Using a Pasteur pipette at least half the volume of semen as for the culture medium was very carefully layered at the bottom of the tube, below the culture medium, without disturbing the interface. The sample was then incubated for at least 20

minutes (37°C, 5% CO<sub>2</sub>), after which the culture medium above the interface, containing the motile sperm, was aspirated and pooled prior to evaluation.

### **3.5.2 PureSperm<sup>®</sup> 40/80 density gradient**

The density gradient centrifugation technique was performed using PureSperm<sup>®</sup> 40/80 (Nidacn, Gothenburg, Sweden). Two millilitres of PureSperm<sup>®</sup> 80 was added to a conical centrifugal tube. This was followed by carefully layering 2 mL PureSperm<sup>®</sup> 40 on top of the denser PureSperm<sup>®</sup> 80 medium. Using a Pasteur pipette, 1.5 mL of semen was layered onto the PureSperm<sup>®</sup>. The tube was then centrifuged at 300 X g for 20 minutes. After centrifugation, the top layer containing less motile sperm in the PureSperm<sup>®</sup> 40 was aspirated and placed into a 5 mL tube which was filled to the 5 mL mark with Ham's F10 medium. The PureSperm<sup>®</sup> 80 medium and the sperm pellet containing more motile cells was collected and resuspended in 5 mL Ham's F10 medium.

The two tubes were centrifuged at 400 X g for 10 minutes. The supernatant was subsequently aspirated, leaving as little liquid as possible above the pellet. The sperm pellet was resuspended in a suitable volume of Ham's-F10 + 3% BSA medium to obtain the required sperm concentration.

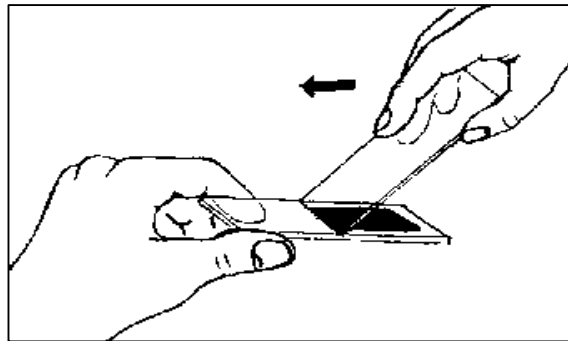
Morphologically normal sperm with dense and homogenous nuclei are expected to concentrate in the denser fraction of the gradient, whereas cells with abnormalities are not expected to migrate similarly. Thus, theoretically, the separation should result in an improvement in the percentage of sperm with normal morphology (Ren *et al.*, 2004).

### **3.6 Slide preparation and staining**

For each stained preparation a drop of semen was spread out gently onto a 76 X 26 mm glass slide. The volume of sample used to make a morphology smear was determined according to the sperm concentration of the given population. According to the 1999 WHO manual, if the sperm concentration is over 20 X 10<sup>6</sup>/mL, then 5µl of



semen can be used; if the sperm concentration is less than  $20 \times 10^6/\text{mL}$ , then 10 to 20  $\mu\text{l}$  of semen should be used. The 'feathering' technique, whereby the edge of a second slide is used to drag a drop of semen along the surface of the slide was employed to make the smears of the spermatozoa, taking care not to make the smears too thick. A thin film of regular thickness ensures optimal visualization of each individual spermatozoon. After air-drying and appropriate fixation, the smears were stained according to the Papanicolaou, SpermBlue<sup>®</sup> and Rapidiff<sup>®</sup> techniques.



*Figure 3. Semen smearing method for sperm morphology (71)*

### **3.6.1 Papanicolaou (PAP) staining technique**

PAP staining was done by experienced technicians in a routine clinical andrology laboratory (Andrology Laboratory, Department of Obstetrics and Gynaecology, Tygerberg Academic Hospital and Stellenbosch University, Tygerberg, South Africa). The chemicals used for the Papanicolaou staining procedure were obtained from Merck, Modderfontein, South Africa. The classic form of the PAP stain involves five dyes in three solutions:

1. Wash with 96 %, 80 %, 70 % and 50 % alcohol respectively
2. Rinse with tap water
3. Stain in Harris' Hematoxylin solution (3 minutes)
4. Rinse with tap water (3-5minutes)

5. Wash with 50 %, 70 %, 80 % and 96 % alcohol respectively
6. Stain with Orange G solution (3 minutes)
7. Wash with 96 % alcohol (repeat)
8. Stain with polychrome staining solution EA31 (3 minutes)
9. Dehydrate with 96 % alcohol (repeat)
10. Dehydrate with absolute alcohol (5 minutes)
11. Dehydrate with equal parts of absolute alcohol and xylene
12. Clear with xylene (2 minutes)

### **3.6.2 Rapidiff<sup>®</sup> (RD) staining technique**

RD is a stain with identical solutions to that of the commonly know DiffQuik<sup>®</sup> stain. The RD staining kit was obtained from Clinical Sciences Diagnostics, Southdale, South Africa. The notable characteristic of this staining technique is its quick and easy methodology, involving only a few short steps.

1. Submerge smear for 6 one-second dips in a RD fixative
2. Submerge smear for six one-second dips in RD Stain 1 (Eosin Y)
3. Continue by submerging the smear in RD Stain 2 (Thiazine Dye Mixture) for 6 one-second dips
4. Finally rinse in a phosphate buffer (pH 6.8) and air-dry

### **3.6.3 SpermBlue<sup>®</sup> (SB) staining technique**

The SB staining kit was supplied by Microptic S.L., Barcelona, Spain.

1. The air-dried smears were carefully placed vertically into a staining tray containing SB fixative, and were left undisturbed for 10 minutes.

2. The slides were then carefully removed from the staining tray and placed at an angle of 60 to 80° to drain off excess fixative. No washing or drying was needed after fixation.
3. The fixed smears were then placed horizontally down onto filter paper, and using a plastic disposable pipette, 0.45 to 0.5 mL of SB stain was dropped onto the fixed sperm smear.
4. The slide was then gently rolled from side to side at regular intervals (approximately once every minute) to ensure that the stain is displaced equally across the smear surface.
5. The stain was left on the slide for 12 – 15 minutes after which the slides were then slowly immersed for 3 seconds in distilled water to remove excess dye. Care had to be taken with immersions as to prevent too many sperm being lost during the rinsing step. The slides were then left in an upright position (at about 70° angle), allowing excess fluid to run off and to air-dry.

### **3.6.4 Mounting**

Following completion of the staining procedures, the morphology slides were mounted using DPX mounting glue and a cover-slip.

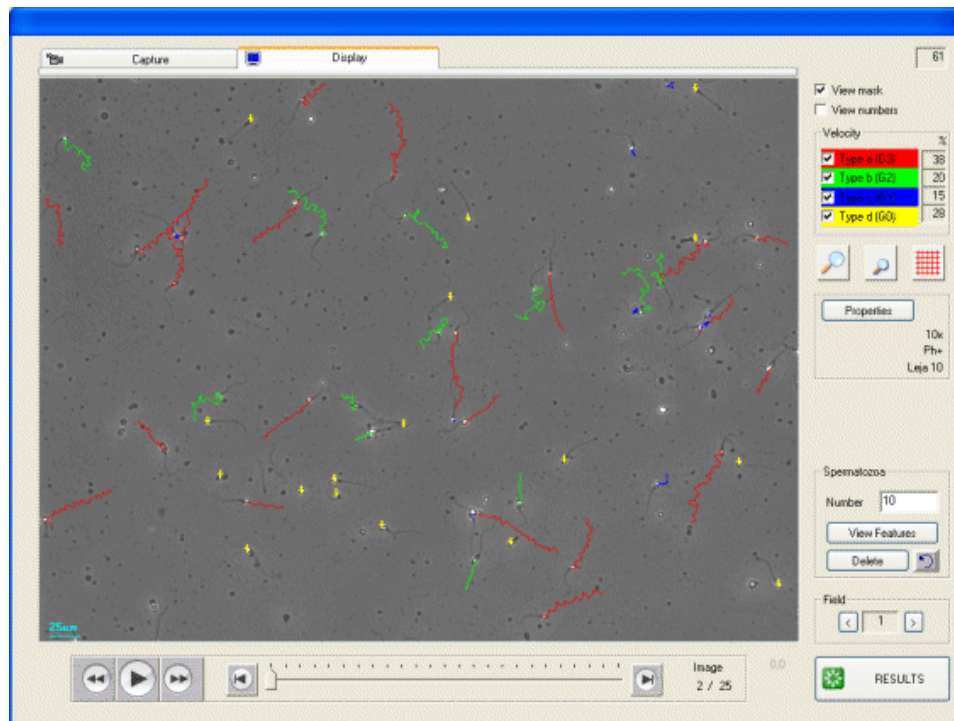
### **3.7 Computer aided semen analysis (CASA)**

The Sperm Class Analyzer<sup>®</sup> (SCA<sup>®</sup>) from the company Microptic S.L., provides fast, accurate and objectively repeatable results that would be impossible to attain using traditional, subjective methods. The SCA<sup>®</sup> was extensively utilized throughout the study for the evaluation of sperm concentration, motility as well as morphology. The system is comprised of a Basler A312fc digital colour camera (Microptic S.L., Barcelona, Spain) with a 780 x 580 pixels resolution and the ability to capture 53 frames per second. The camera was mounted (C-mount) on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa), equipped with bright field optics. The

Basler camera was connected via a six pin FireWire cable (IEEE1394) to a Belkin firewire card of a desktop computer. A temperature-regulated microscope stage allowed for the accurate measurement of motility. The aforementioned set-up along with the *SCA<sup>®</sup> Motility and Concentration* and *SCA<sup>®</sup> Morphology* modules were utilised throughout the study.

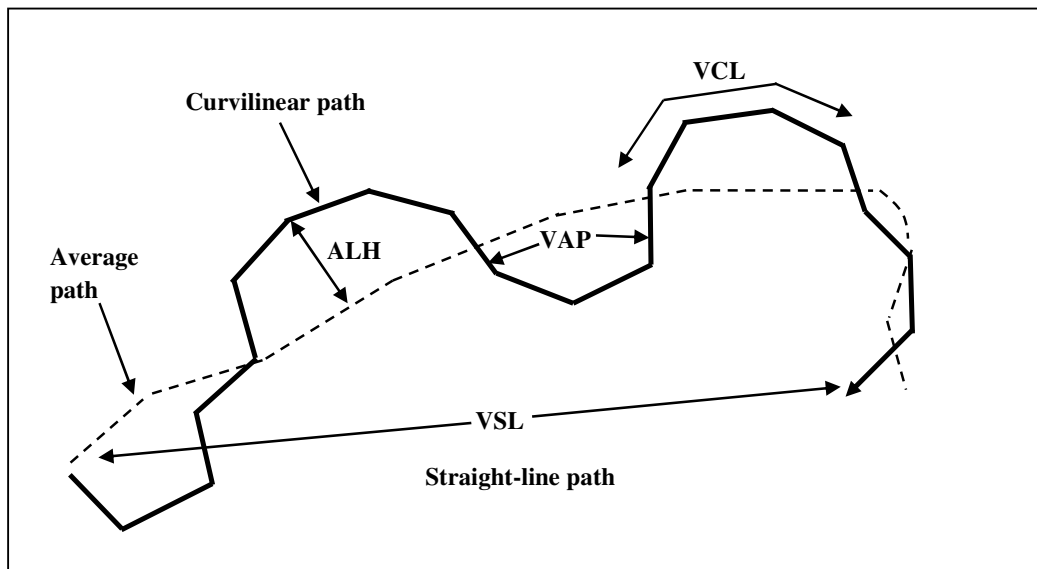
### 3.7.1 *SCA<sup>®</sup> Motility and concentration*

For motility analysis, approximately 2µl of semen was loaded into a single chamber of a Leja<sup>®</sup> four-chamber slide with a 20µm depth, and this was subsequently analysed using a x40 objective. *SCA<sup>®</sup> Motility and Concentration* provides automatic, immediate and objective detailed results of motility and concentration in a complete report. The software detects the motile (type a, b and c) and immotile (type d) spermatozoa automatically, whilst performing an accurate count and concentration measurement.



**Figure 4:** Screenshot of visualisation following the analysis of sperm concentration and motility using the *SCA<sup>®</sup>*. The different colour paths indicate whether the sperm is classified as type a (red), type b (green), type c (blue) or type d (yellow) (69).

The motility analyses are performed by following the sperm trajectory and subsequently determines velocity (*See Figure 5*). The optical and SCA<sup>®</sup> software settings were as follows: optics, Ph+; contrast, 435; brightness, 100; scale, x10; chamber, Leja 20; capture 50 images per second; curvilinear velocity [VCL],  $10\mu\text{m/s} < \text{slow} < 15\mu\text{m/s}$ ,  $15\mu\text{m/s} < \text{medium} < 35\mu\text{m/s}$ ,  $35\mu\text{m/s} < \text{rapid}$ ; progressivity,  $>80\%$  of STR; circular,  $<50\%$  linearity [LIN]; connectivity, 12; low average path velocity [VAP] points,  $5\mu\text{m/s}$ ; and temperature,  $37^\circ\text{C}$  and hyperactivity,  $3.75 \leq x \leq 15$  [ALH],  $1 \leq x \leq 5$  [LIN] and  $150 \leq x \leq 500$  [VCL].



**Figure 5:** An illustration of different sperm motility parameters using CASA (111)

Motility parameters analyzed by means of SCA<sup>®</sup> include the following:

- (i) Motility: the percentage of motile spermatozoa consisting of type a, b and c sperm possessing  $\geq 25\mu\text{m/s}$  progressive,  $< 25\mu\text{m/s}$  progressive or  $< 5\mu\text{m/s}$  non-progressive motility respectively
- (ii) Progressive motility: the percentage of progressively motile cells consisting of type a and b sperm
- (iii) Curvilinear velocity (VCL) ( $\mu\text{m/s}$ ): time-average velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions under the microscope

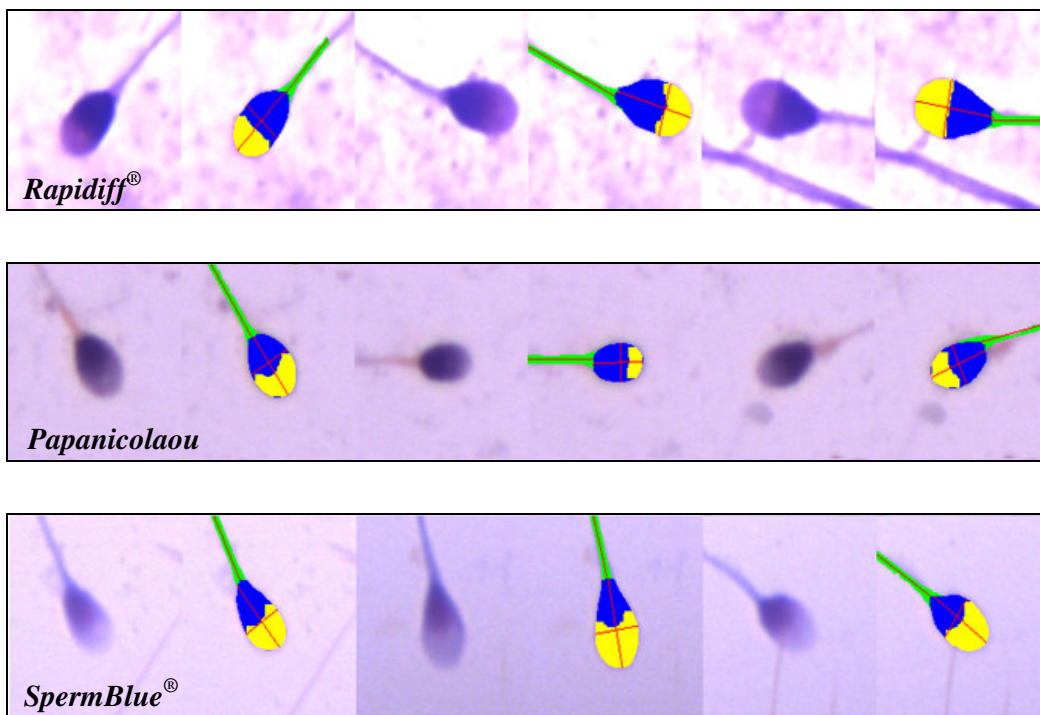
- (iv) Straight line velocity (VSL) ( $\mu\text{m/s}$ ): time-average velocity of a sperm head along the straight line between its first detected position and its last
- (v) Average path velocity (VAP) ( $\mu\text{m/s}$ ): time-average velocity of a sperm head along its average path
- (vi) Amplitude of lateral head displacement (ALH) ( $\mu\text{m}$ ): magnitude of lateral displacement of sperm head about its average path
- (vii) Linearity (LIN): the linearity of a curvilinear path,  $\text{VSL/VCL}$
- (viii) Straightness (STR): linearity of the average path,  $\text{VAP/VCL}$
- (ix) Beat-cross frequency (BCF) (beats/second): the average rate at which the sperm's curvilinear path crosses its average path
- (x) Rapid cells: the percentage of rapidly moving cells
- (xi) Static cells: the percentage of static/motion-less cells

### **3.7.2 SCA<sup>®</sup> Morphology**

For morphology evaluation, the stained sperm were analysed using a blue filter, a x100 oil immersion objective and a x10 eyepiece. Morphological evaluation was performed in several systematically selected areas of the slide. Overlapping spermatozoa and those lying with the head on the edge were excluded as they were not possible to assess. A complete evaluation comprises the morphological evaluation of 100 cells per slide. The brightness and contrast settings in the SCA<sup>®</sup> system were identical for all analyses however, the light settings of the microscope were adjusted accordingly for each staining method to allow for optimal illumination. During the analyses, spermatozoa were analysed at random on different areas of the slide. Fields were not analysed where sperm were found to overlap, or where background staining interfered with the boundary of the sperm head. After the analysis of each sperm, the image and corresponding analysis mask were manually compared, and sperm with

obvious incorrect analyses (i.e. an acrosome depicted in the midpiece region) were eliminated. A total of 100 sperm per slide were analysed.

The SCA<sup>®</sup> automatically and rapidly detects the head and acrosome regions of the stained sperm and analyses the morphometry. For each sperm analysed, the actual stained sperm is shown on the left and on the right the analysis of the same sperm is represented, and masked by areas coloured in yellow, blue and green for the acrosome, head and midpiece regions respectively (See Figure 6).



**Figure 6.** The above images depict the SCA<sup>®</sup> morphology analysis of the same semen sample stained according to Rapidiff<sup>®</sup>, Papanicolaou and SpermBlue<sup>®</sup>. The SCA<sup>®</sup> system recognizes the acrosome (yellow), head (blue) and midpiece (green). Each stained sperm is shown on the left and to its immediate right the SCA<sup>®</sup> analysis of that particular sperm is shown.

For the purpose of this study, only head- and acrosome-related morphometric parameters were considered. Morphometric parameters measured included head

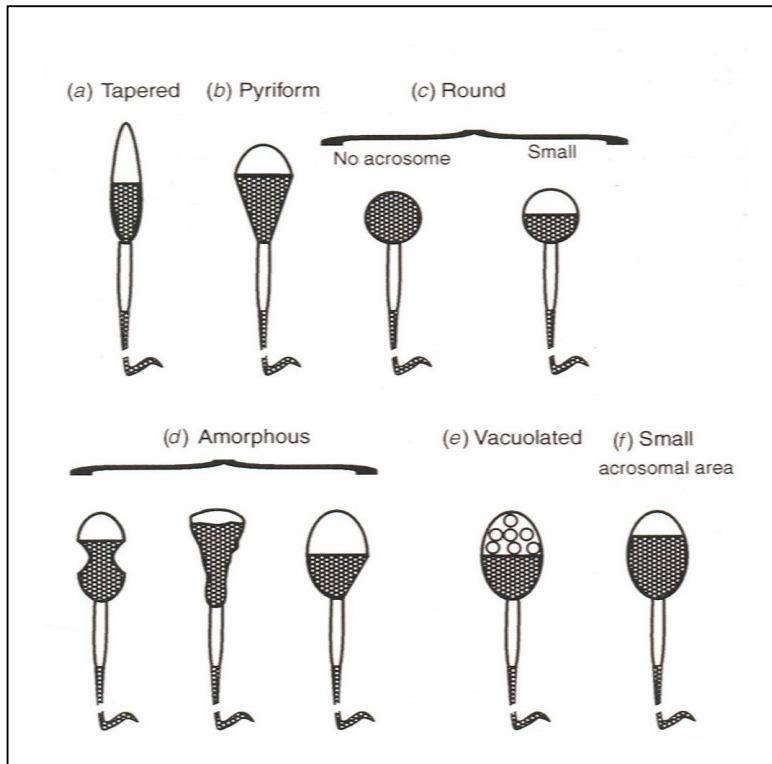
length, -width, -area, -perimeter, -ellipticity, -elongation, -roughness, -regularity and acrosome coverage (*See Table III*). The four software-calculated indexes, namely ellipticity, elongation, roughness and regularity takes into account the standard morphometric measurements and gives an indication of the sperm head shape. For instance, ellipticity indicates if the sperm head is thin or tapered. If the value for head ellipticity is high, this means that the sperm head is thin. Elongation indicates the roundness of the sperm head, where the closer this value is to zero, the rounder the head. The roughness index indicates amorphous or irregular heads for low values, while the regularity index indicates pyriform sperm heads.



**Table III:** Formulas used in the calculation of sperm morphometry measurements (L: length; W: width; A: area; P: perimeter)

<b><i>Morphometric parameter</i></b>	<b><i>Formula</i></b>
Head length ( $\mu\text{m}$ )	L
Head width ( $\mu\text{m}$ )	W
Head area ( $\mu\text{m}^2$ )	A
Head perimeter ( $\mu\text{m}$ )	P
Head ellipticity	L/W
Head elongation	$(L-W)/(L+W)$
Head roughness	$4\pi(A/P^2)$
Head regularity	$\pi(LW/4A)$

Based on the morphometric measurements, the SCA<sup>®</sup> provides morphologic results, which include total normal and abnormal sperm head forms. Additional information regarding the breakdown of normal and abnormal sperm head morphologies are also provided and these include the classing of sperm heads into different morphology categories (See Figure 7). An added advantage of using the SCA<sup>®</sup>, is the option to easily analyse sperm morphology according to both Tygerberg strict criteria and WHO Criteria by adjusting the appropriate settings.



**Figure 7a-f:** Morphology categories of sperm head defects (111)

### 3.8 Statistical evaluation

GraphPad™ Prism 5 was used for all statistical evaluations. For comparative analyses, Student's t-test (unpaired) or one-way analysis of variance (ANOVA) tests (with Bonferroni *post hoc* test if  $P < 0.005$ ) were used. Results are expressed as the mean  $\pm$  standard error of the mean. Findings are considered statistically significant when  $p < 0.05$ .

## CHAPTER 4

### RESULTS

All statistical results obtained during the course of the investigation are provided in this chapter. Results are presented in the form of bar graphs displaying the standard error of the mean and significance bars where applicable, followed by a description of statistically significant results. Complete tables containing the measurements for all sperm parameters captured by the SCA<sup>®</sup> during the investigation are presented at the end of each subsection (*See Tables IV- XII*). Although statistical analyses for each set of parameters have been performed and provided in these tables, due to the scope of the topic not all parameters will form part of the discussion in this dissertation, and have merely been included for the reader's interest and to provide information that may prove to be useful in future investigations. Those parameters which are accompanied by bar-graphs will form part of the main discussion.

#### **4.1 The influence of three different morphology staining techniques on human sperm head morphometry and morphology**

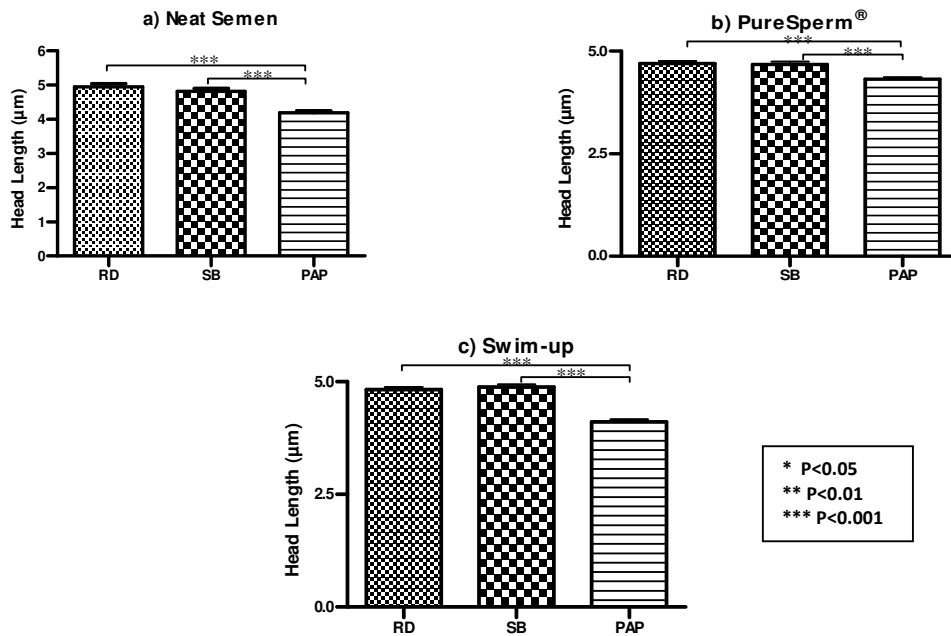
In order to investigate the effects different staining techniques might have on sperm head dimensions, we examined the differences in various sperm morphometry parameters using a neat semen smear, along with semen which has been processed using the swim-up and PureSperm<sup>®</sup> density gradient separation methods. The aim in this section of the study was to identify any possible shortcomings surrounding the lack of standardization regarding the use of various morphology stains during sperm morphology analysis.

##### **4.1.1 Sperm head morphometry**

###### ***Head Length***

Results showed that sperm stained with RD had the largest values for head length, followed by those stained with SB and then PAP (RD>SB>PAP). The results for head length showed a significant difference between sperm stained with RD and PAP in all

the sperm fractions (Fig. 8a: RD vs. PAP:  $4.943\mu\text{m} \pm 0.094\mu\text{m}$  vs.  $4.189\mu\text{m} \pm 0.059\mu\text{m}$ ;  $p < 0.001$ ; Fig. 8b: RD vs. PAP:  $4.701\mu\text{m} \pm 0.068\mu\text{m}$  vs.  $4.230\mu\text{m} \pm 0.047\mu\text{m}$ ;  $p < 0.001$  and Fig. 8c: RD vs. PAP:  $4.821\mu\text{m} \pm 0.065\mu\text{m}$  vs.  $4.106\mu\text{m} \pm 0.059\mu\text{m}$   $p < 0.001$ ). In all populations SB-stained sperm was significantly greater than sperm stained using PAP (Fig. 8a: SB vs. PAP:  $4.808\mu\text{m} \pm 0.084\mu\text{m}$  vs.  $4.189\mu\text{m} \pm 0.059\mu\text{m}$ ;  $p < 0.001$ ; Fig. 8b: SB vs. PAP:  $4.681\mu\text{m} \pm 0.063\mu\text{m}$  vs.  $4.230\mu\text{m} \pm 0.047\mu\text{m}$ ;  $p < 0.001$  and Fig. 8c:  $4.886\mu\text{m} \pm 0.056\mu\text{m}$  vs.  $4.106\mu\text{m} \pm 0.059\mu\text{m}$ ;  $p < 0.001$ ).

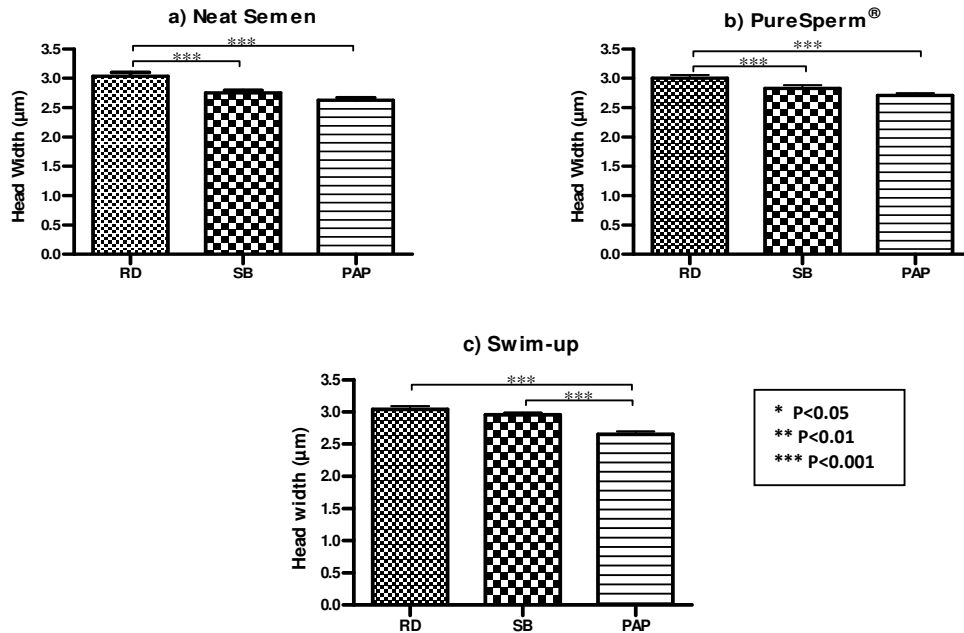


**Figure 8a-c.** Effects of different morphology stains on sperm head length (n=20)

### Head Width

Results across the three populations investigated consistently showed that head width was largest among the RD-stained sperm, followed by those stained by SB and PAP respectively (RD>SB>PAP). Significant differences were found between RD and PAP in all the populations (Fig. 9a: RD vs. Pap:  $3.034\mu\text{m} \pm 0.064\mu\text{m}$  vs.  $2.752\mu\text{m} \pm 0.046\mu\text{m}$ ;  $p < 0.001$ ; Fig. 9b: RD vs. PAP:  $3.003\mu\text{m} \pm 0.052\mu\text{m}$  vs.  $2.825\mu\text{m} \pm 0.053\mu\text{m}$ ;  $p < 0.05$ ; Fig. 9c: RD vs. PAP:  $3.040\mu\text{m} \pm 0.047\mu\text{m}$  vs.  $2.652\mu\text{m} \pm 0.042\mu\text{m}$ ;  $p < 0.001$ ). A significant difference was found between RD- and SB-stained sperm in

the neat and PureSperm<sup>®</sup> density gradient fractions (Fig. 9a: RD vs. SB:  $3.034 \mu\text{m} \pm 0.064 \mu\text{m}$  vs.  $2.627 \mu\text{m} \pm 0.046 \mu\text{m}$ ;  $p < 0.001$  and Fig. 9b: RD vs. SB:  $3.003 \mu\text{m} \pm 0.052 \mu\text{m}$  vs.  $2.711 \mu\text{m} \pm 0.039 \mu\text{m}$ ;  $p < 0.001$ ), whereas a significant difference between SB- and PAP-stained sperm was evident in the Swim-up fraction (Fig. 9c: SB vs. PAP:  $2.951 \mu\text{m} \pm 0.043 \mu\text{m}$  vs.  $2.652 \mu\text{m} \pm 0.042 \mu\text{m}$ ;  $p < 0.001$ ).

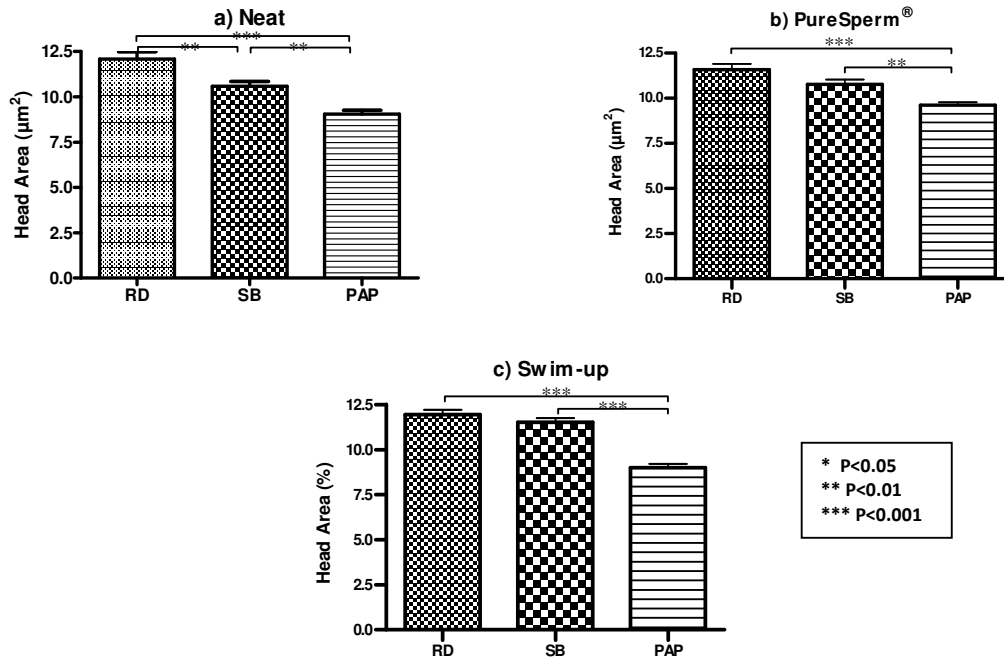


**Figure 9a-c.** Effects of different morphology stains on sperm head width ( $n=20$ )

### Head Area

As with head length and head width, results showed that RD-stained sperm possessed the highest values for head area, followed by SB- and PAP-stained sperm respectively. All populations showed a significant difference between sperm stained with RD and PAP (Fig. 10a: RD vs. PAP:  $12.090 \mu\text{m}^2 \pm 0.411 \mu\text{m}^2$  vs.  $9.043 \mu\text{m}^2 \pm 0.227 \mu\text{m}^2$ ;  $P < 0.01$ ; Fig. 10b: RD vs. PAP:  $11.580 \mu\text{m}^2 \pm 0.303 \mu\text{m}^2$  vs.  $9.607 \mu\text{m}^2 \pm 0.177 \mu\text{m}^2$ ;  $P < 0.001$  and Fig. 10c: RD vs. PAP:  $11.940 \mu\text{m}^2 \pm 0.285 \mu\text{m}^2$  vs.  $8.988 \mu\text{m}^2 \pm 0.215 \mu\text{m}^2$ ;  $P < 0.001$ ), as well as between the SB and PAP-stained sperm (Fig. 10a: SB vs. PAP:  $10.580 \mu\text{m}^2 \pm 0.276 \mu\text{m}^2$  vs.  $9.043 \mu\text{m}^2 \pm 0.227 \mu\text{m}^2$ ;  $p < 0.001$ ; Fig. 10b: SB vs. PAP:  $10.760 \mu\text{m}^2 \pm 0.272 \mu\text{m}^2$  vs.  $9.607 \mu\text{m}^2 \pm 0.177 \mu\text{m}^2$ ;  $p < 0.01$  and

Fig. 10c: SB vs. PAP:  $11.520 \mu\text{m}^2 \pm 0.224 \mu\text{m}^2$  vs.  $8.988 \mu\text{m}^2 \pm 0.215 \mu\text{m}^2$ ;  $p < 0.001$ ). In the neat fraction, a significant difference in head area was evident between RD- and SB-stained sperm (Fig. 10a: RD vs. SB:  $12.090 \mu\text{m}^2 \pm 0.411 \mu\text{m}^2$  vs.  $10.580 \mu\text{m}^2 \pm 0.276 \mu\text{m}^2$ ;  $p < 0.01$ ) was also shown.

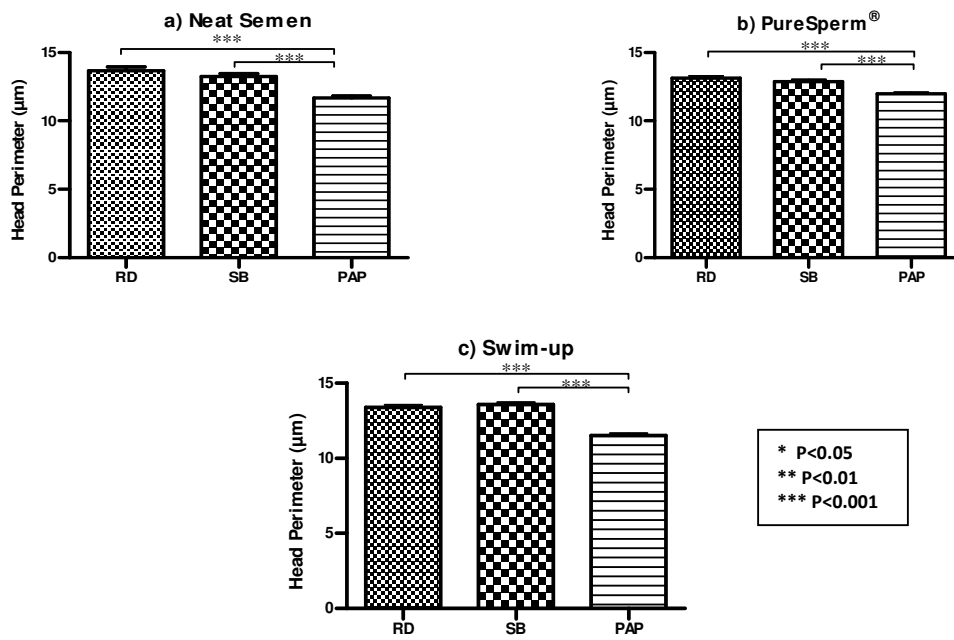


**Figure 10a-c.** Effects of different morphology stains on sperm head area ( $n=20$ )

### Head Perimeter

The comparison of head perimeter revealed that head perimeters were greatest with sperm stained with RD, followed by SB and PAP (RD<SB<PAP), with significant differences found between RD- and PAP-stained sperm (Fig. 11a: RD vs. PAP:  $13.690 \mu\text{m} \pm 0.268 \mu\text{m}$  vs.  $11.670 \mu\text{m} \pm 0.159 \mu\text{m}$ ;  $p < 0.001$ ; Fig. 11b: RD vs. PAP:  $13.130 \mu\text{m} \pm 0.181 \mu\text{m}$  vs.  $11.990 \mu\text{m} \pm 0.115 \mu\text{m}$ ;  $p < 0.001$  and Fig. 11c: RD vs. PAP:  $13.380 \mu\text{m} \pm 0.169 \mu\text{m}$  vs.  $11.500 \mu\text{m} \pm 0.151 \mu\text{m}$ ;  $p < 0.001$ ), and with SB- and PAP-stained sperm (Fig. 11a: SB vs. PAP:  $13.230 \mu\text{m} \pm 0.226 \mu\text{m}$  vs.  $11.670 \mu\text{m} \pm 0.159 \mu\text{m}$ ;  $p < 0.001$ ; Fig. 11b: SB vs. PAP:  $12.860 \mu\text{m} \pm 0.167 \mu\text{m}$  vs.  $11.990 \mu\text{m} \pm$

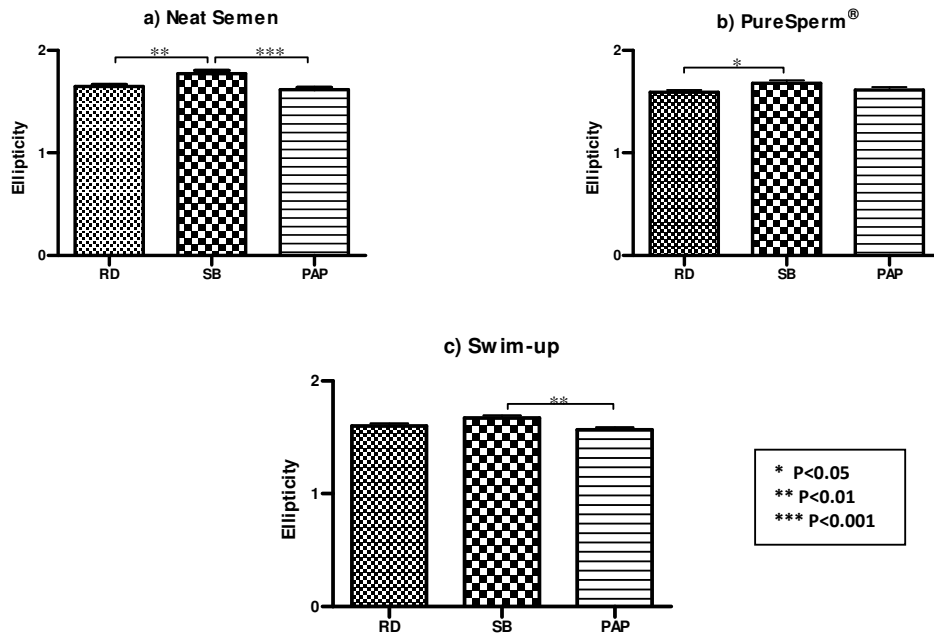
0.115  $\mu\text{m}$  and Fig. 11c: SB vs. PAP: 13.580  $\mu\text{m} \pm 0.166 \mu\text{m}$  vs. 11.500  $\mu\text{m} \pm 0.151 \mu\text{m}$ ;  $p < 0.001$ ) in all populations.



**Figure 11a-c.** Effects of different morphology stains on sperm head perimeter ( $n=20$ )

### Head Ellipticity

Values for head ellipticity were found to be highest in the SB-stained sperm, with significant differences found in the neat and PureSperm® density gradient populations between RD and SB (Fig. 12a: SB vs. RD:  $1.774 \pm 0.032$  vs.  $1.645 \pm 0.020$ ;  $p < 0.01$ ; Fig. 12b: SB vs. RD:  $1.679 \pm 0.027$  vs.  $1.590 \pm 0.022$ ;  $p < 0.05$ ). SB-stained sperm head ellipticity showed to be significantly higher than sperm staining using PAP in the neat and swim-up populations (Fig. 12a: SB vs. PAP:  $1.774 \pm 0.032$  vs.  $1.614 \pm 0.023$ ;  $p < 0.001$  and Fig. 12c: SB vs. PAP:  $1.673 \pm 0.023$  vs.  $1.567 \pm 0.023$ ;  $p < 0.01$ ).

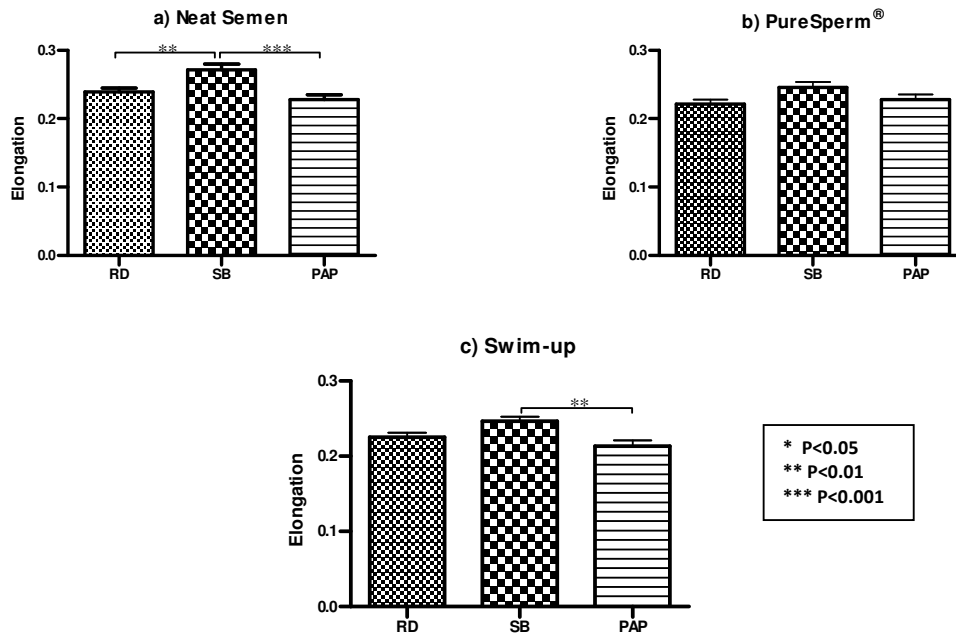


**Figure 12a-c.** Effects of different morphology stains on sperm head ellipticity (n=20)

### **Head Elongation**

Sperm stained using RD and PAP stains revealed similar values for head elongation. However, there were significant differences found in the neat fraction between SB- and RD-stained sperm (Fig. 13a: SB vs. RD:  $0.272 \pm 0.083$  vs.  $0.239 \pm 0.006$ ;  $p < 0.01$ ) and SB and PAP-stained sperm (Fig. 13a: SB vs. PAP:  $0.272 \pm 0.083$  vs.  $0.228 \pm 0.007$ ;  $p < 0.001$ ) respectively. No significant differences were observed in the PureSperm® density gradient fraction, however in the swim-up population there was a significant difference between sperm stained with SB and PAP (Fig. 13c: SB vs. PAP:  $0.246 \pm 0.007$  vs.  $0.214 \pm 0.007$ ;  $p < 0.01$ ).

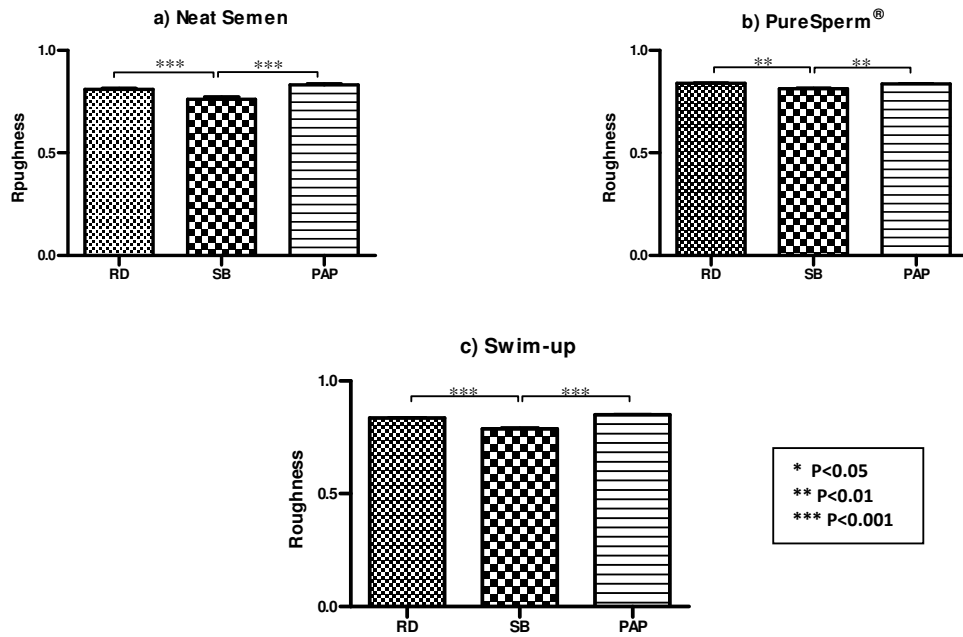




**Figure 13a-c.** Effects of different morphology stains on sperm head elongation (n=20)

### Head Roughness

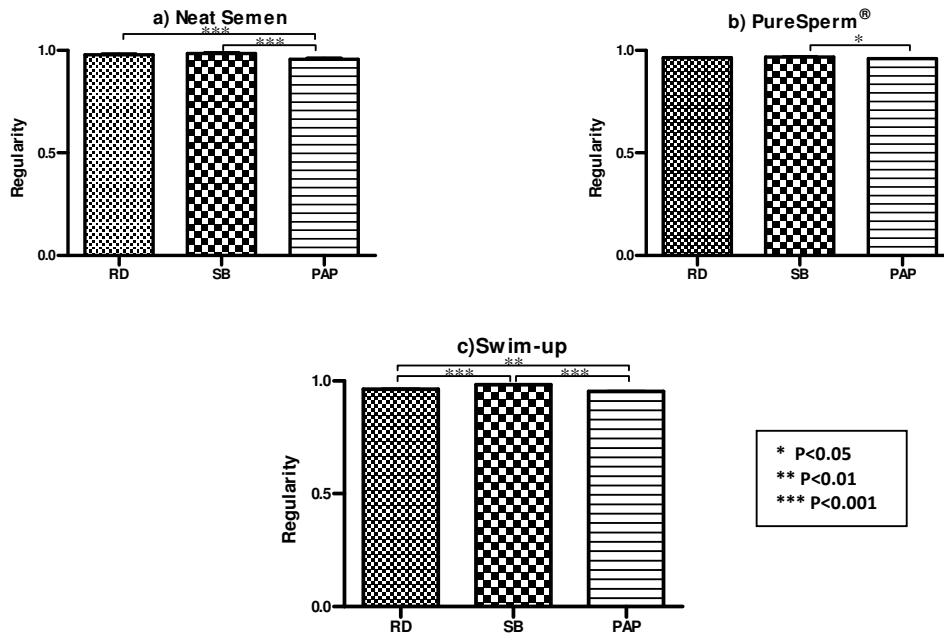
In all the populations investigated, head roughness was found to be smallest in the SB-population, with RD- and PAP-stained sperm having similar values. In all populations a significant difference was found between SB- and RD-stained sperm (Fig. 14a: RD vs. SB:  $0.810 \pm 0.005$  vs.  $0.762 \pm 0.010$ ;  $p < 0.001$ , Fig 14b: RD vs. SB:  $0.840 \pm 0.004$  vs.  $0.814 \pm 0.006$ ;  $p < 0.001$  and Fig. 14c: RD vs. SB:  $0.837 \pm 0.004$  vs.  $0.788 \pm 0.007$ ;  $p < 0.001$ ), and SB- and PAP-stained sperm (Fig. 14a: PAP vs. SB:  $0.836 \pm 0.005$  vs.  $0.762 \pm 0.010$ ;  $p < 0.001$ , Fig. 14b: PAP vs. SB:  $0.836 \pm 0.005$  vs.  $0.814 \pm 0.006$ ;  $p < 0.001$  and Fig. 14c: PAP vs. SB:  $0.851 \pm 0.005$  vs.  $0.788 \pm 0.007$ ;  $p < 0.001$ ) respectively.



**Figure 14a-c.** Effects of different morphology stains on sperm head roughness (n=20)

### **Head Regularity**

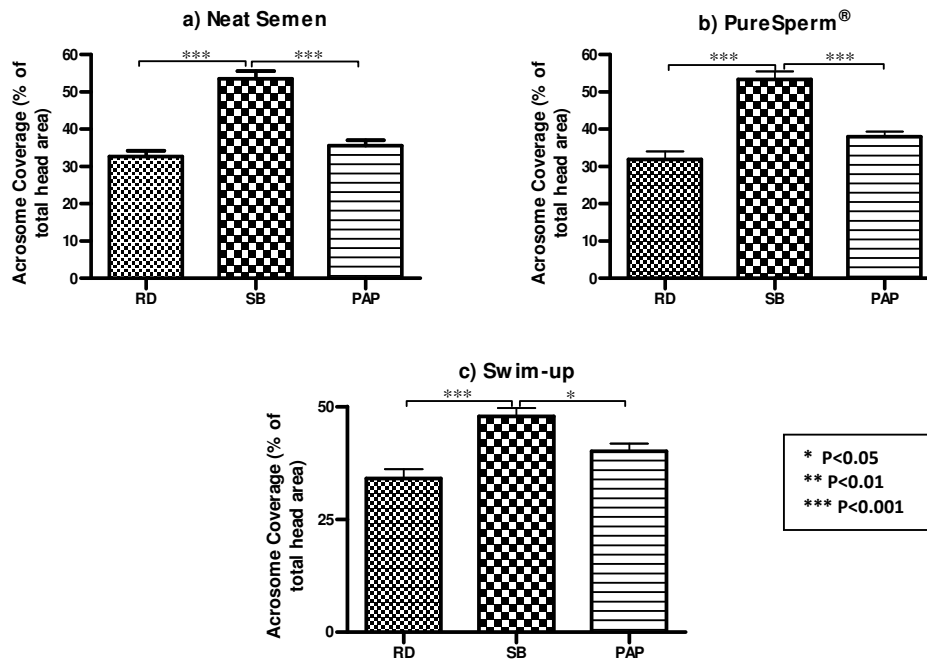
Head regularity was shown to be highest among the SB-stained sperm in all the populations investigated. A significant difference was found between sperm stained using SB and PAP in the neat (Fig. 15a: SB vs. PAP:  $0.984 \pm 0.004$  vs.  $0.957 \pm 0.003$ ;  $p < 0.001$ ), PureSperm® density gradient (Fig. 15b: SB vs. PAP:  $0.968 \pm 0.003$  vs.  $0.958 \pm 0.002$ ;  $p < 0.05$ ) and swim-up (Fig. 15c: SB vs. PAP:  $0.984 \pm 0.002$  vs.  $0.954 \pm 0.003$ ;  $p < 0.001$ ) populations. Significance was also found between RD- and PAP-stained sperm in the neat (Fig. 15a: RD vs. PAP:  $0.978 \pm 0.004$  vs.  $0.957 \pm 0.003$ ;  $p < 0.001$ ) and swim-up (Fig. 15c: RD vs. PAP:  $0.965 \pm 0.002$  vs.  $0.954 \pm 0.003$ ;  $p < 0.01$ ) populations. Additionally, significance was evident in the swim-up population between RD- and SB-stained sperm (Fig. 15c: SB vs. RD:  $0.965 \pm 0.002$  vs.  $0.984 \pm 0.002$ ;  $p < 0.001$ ).



**Figure 15a-c.** Effects of different morphology stains on sperm head regularity (n=20)

### **Acrosome Coverage**

Results concerning acrosome coverage were found to be consistent among the populations, where SB-stained sperm were shown to possess the largest percentage acrosome coverage, followed by PAP- and RD-stained sperm respectively (SB>PAP>RD). Sperm stained using SB showed to be significantly higher than those stained with PAP (Fig. 16a: SB vs. PAP: 53.508% ± 2.005% vs. 35.640% ± 1.411%; p<0.001; Fig. 16b: SB vs. PAP: 53.350% ± 2.121% vs. 37.990% ± 1.412% and Fig. 16c: SB vs. PAP: 47.830% ± 1.848% vs. 40.130% ± 1.697%; p<0.05) and RD (Fig. 16a: SB vs. RD: 53.580% ± 2.005% vs. 32.580% ± 1.616%; p<0.001; Fig. 16b: SB vs. RD: 53.350% ± 2.121% vs. 31.840% ± 2.185%; p<0.001 and Fig. 16c: SB vs. RD: 47.830% ± 1.848% vs. 34.070% ± 2.105%; p<0.001) in all the populations investigated.



**Figure 16a-c.** Effects of different morphology stains on acrosome coverage (n=20)

**Table IV:** The effects of different morphology stains on sperm head morphometry in neat semen (Mean ± SEM) (n=20)

	<i>Rapidiff</i> ®	<i>SpermBlue</i> ®	<i>Papanicolaou</i>
Length (µm)	4.943 ± 0.094	4.808 ± 0.084 <sup>bbb</sup>	4.189 ± 0.059 <sup>ccc</sup>
Width (µm)	3.034 ± 0.064 <sup>aaa</sup>	2.752 ± 0.046	2.627 ± 0.044 <sup>ccc</sup>
Area (µm <sup>2</sup> )	12.090 ± 0.411 <sup>aa</sup>	10.580 ± 0.276 <sup>bb</sup>	9.043 ± 0.227 <sup>ccc</sup>
Perimeter (µm)	13.690 ± 0.268	13.230 ± 0.226 <sup>bbb</sup>	11.670 ± 0.159 <sup>ccc</sup>
Ellipticity	1.645 ± 0.020 <sup>aa</sup>	1.774 ± 0.032 <sup>bbb</sup>	1.614 ± 0.023
Elongation	0.239 ± 0.006 <sup>aa</sup>	0.272 ± 0.008 <sup>bbb</sup>	0.228 ± 0.007
Roughness	0.810 ± 0.005 <sup>aaa</sup>	0.762 ± 0.010 <sup>bbb</sup>	0.832 ± 0.004
Regularity	0.978 ± 0.004	0.984 ± 0.004 <sup>bbb</sup>	0.957 ± 0.003 <sup>ccc</sup>
Acrosome (%)	32.580 ± 1.616 <sup>aaa</sup>	53.580 ± 2.005 <sup>bbb</sup>	35.640 ± 1.411

<sup>a</sup> *Rapidiff*® vs. *SpermBlue*® P<0.05; <sup>aa</sup> *Rapidiff*® vs. *SpermBlue*® P<0.01; <sup>aaa</sup> *Rapidiff*® vs. *SpermBlue*® P<0.001

<sup>b</sup> *SpermBlue*® vs. *Papanicolaou* P<0.05; <sup>bb</sup> *SpermBlue*® vs. *Papanicolaou* P<0.01; <sup>bbb</sup> *SpermBlue*® vs. *Papanicolaou* P<0.001

<sup>c</sup> *Papanicolaou* vs. *Rapidiff*® P<0.05; <sup>cc</sup> *Papanicolaou* vs. *Rapidiff*® P<0.01; <sup>ccc</sup> *Papanicolaou* vs. *Rapidiff*® P<0.001

**Table V:** The effects of different morphology stains on head morphometry in sperm obtained via the PureSperm<sup>®</sup> density gradient separation technique (Mean ± SEM) (n=20)

	<i>Rapidiff</i> <sup>®</sup>	<i>SpermBlue</i> <sup>®</sup>	<i>Papanicolaou</i>
Length (µm)	4.701 ± 0.068	4.681 ± 0.063 <sup>bbb</sup>	4.230 ± 0.047 <sup>ccc</sup>
Width (µm)	3.003 ± 0.052 <sup>a</sup>	2.825 ± 0.053	2.711 ± 0.039 <sup>ccc</sup>
Area (µm <sup>2</sup> )	11.580 ± 0.303	10.760 ± 0.272 <sup>bb</sup>	9.607 ± 0.177 <sup>ccc</sup>
Perimeter (µm)	13.130 ± 0.181	12.860 ± 0.167 <sup>bbb</sup>	11.990 ± 0.115 <sup>ccc</sup>
Ellipticity	1.590 ± 0.022 <sup>a</sup>	1.679 ± 0.0273	1.614 ± 0.026
Elongation	0.222 ± 0.006	0.246 ± 0.0078	0.228 ± 0.008
Roughness	0.840 ± 0.004 <sup>aaa</sup>	0.814 ± 0.006 <sup>bbb</sup>	0.836 ± 0.005
Regularity	0.965 ± 0.003	0.968 ± 0.003 <sup>b</sup>	0.958 ± 0.002
Acrosome (%)	31.840 ± 2.185 <sup>aaa</sup>	53.350 ± 2.121 <sup>bbb</sup>	37.990 ± 1.412
<sup>a</sup> Rapidiff <sup>®</sup> vs. SpermBlue <sup>®</sup> P<0.05; <sup>aa</sup> Rapidiff <sup>®</sup> vs. SpermBlue <sup>®</sup> P<0.01; <sup>aaa</sup> Rapidiff <sup>®</sup> vs. SpermBlue <sup>®</sup> P<0.001 <sup>b</sup> SpermBlue <sup>®</sup> vs. Papanicolaou P<0.05; <sup>bb</sup> SpermBlue <sup>®</sup> vs. Papanicolaou P<0.01; <sup>bbb</sup> SpermBlue <sup>®</sup> vs. Papanicolaou P<0.001 <sup>c</sup> Papanicolaou vs. Rapidiff <sup>®</sup> P<0.05; <sup>cc</sup> Papanicolaou vs. Rapidiff <sup>®</sup> P<0.01; <sup>ccc</sup> Papanicolaou vs. Rapidiff <sup>®</sup> P<0.001			

**Table VI:** The effects of different morphology stains on head morphometry in sperm obtained via the swim-up method (Mean ± SEM) (n=20)

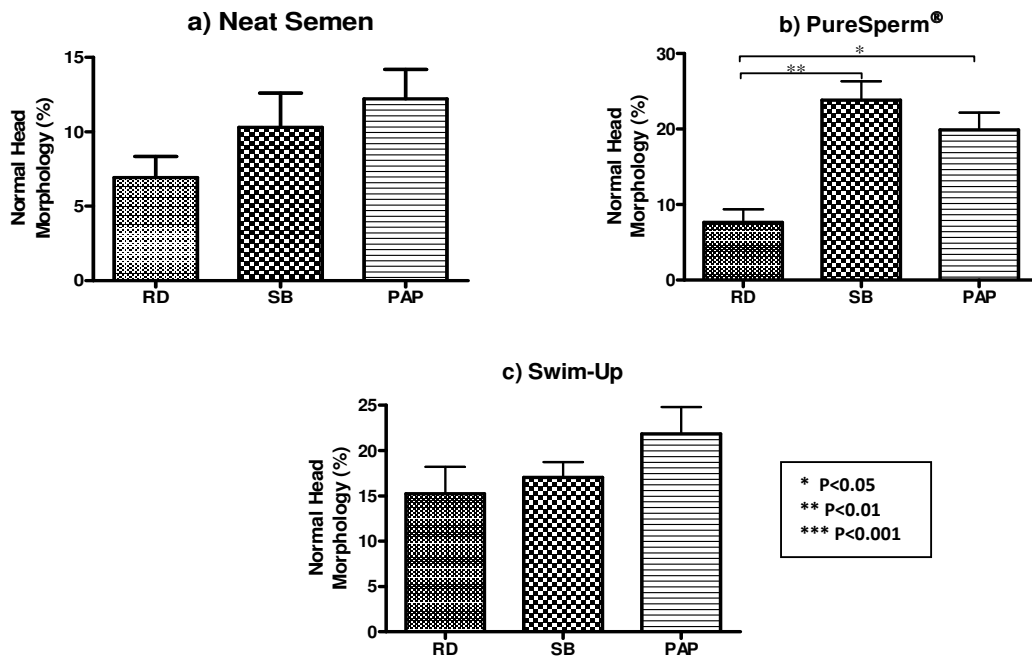
	<i>Rapidiff</i> <sup>®</sup>	<i>SpermBlue</i> <sup>®</sup>	<i>Papanicolaou</i>
Length (µm)	4.821 ± 0.065	4.886 ± 0.056 <sup>bbb</sup>	4.106 ± 0.059 <sup>ccc</sup>
Width (µm)	3.040 ± 0.047	2.951 ± 0.043 <sup>bbb</sup>	2.652 ± 0.042 <sup>ccc</sup>
Area (µm <sup>2</sup> )	11.940 ± 0.285	11.520 ± 0.224 <sup>bbb</sup>	8.988 ± 0.215 <sup>ccc</sup>
Perimeter (µm)	13.380 ± 0.169	13.580 ± 0.166 <sup>bbb</sup>	11.500 ± 0.151 <sup>ccc</sup>
Ellipticity	1.601 ± 0.022	1.673 ± 0.023 <sup>bb</sup>	1.567 ± 0.023
Elongation	0.225 ± 0.006	0.246 ± 0.007 <sup>bb</sup>	0.214 ± 0.007
Roughness	0.837 ± 0.004 <sup>aaa</sup>	0.788 ± 0.007 <sup>bbb</sup>	0.851 ± 0.005
Regularity	0.965 ± 0.002 <sup>aaa</sup>	0.984 ± 0.002 <sup>bbb</sup>	0.954 ± 0.003 <sup>cc</sup>
Acrosome (%)	34.070 ± 2.105 <sup>aaa</sup>	47.830 ± 1.848 <sup>b</sup>	40.130 ± 1.697
<sup>a</sup> Rapidiff <sup>®</sup> vs. SpermBlue <sup>®</sup> P<0.05; <sup>aa</sup> Rapidiff <sup>®</sup> vs. SpermBlue <sup>®</sup> P<0.01; <sup>aaa</sup> Rapidiff <sup>®</sup> vs. SpermBlue <sup>®</sup> P<0.001 <sup>b</sup> SpermBlue <sup>®</sup> vs. Papanicolaou P<0.05; <sup>bb</sup> SpermBlue <sup>®</sup> vs. Papanicolaou P<0.01; <sup>bbb</sup> SpermBlue <sup>®</sup> vs. Papanicolaou P<0.001 <sup>c</sup> Papanicolaou vs. Rapidiff <sup>®</sup> P<0.05; <sup>cc</sup> Papanicolaou vs. Rapidiff <sup>®</sup> P<0.01; <sup>ccc</sup> Papanicolaou vs. Rapidiff <sup>®</sup> P<0.001			

### 4.1.2 Sperm head morphology

To investigate whether the use of a particular stain affects sperm morphology evaluation, we compared the outcomes of morphology analyses following three different staining techniques. As with the morphometry investigation, we investigated the effects in the neat, as well as the sperm populations isolated by the PureSperm<sup>®</sup> density gradient and swim-up methods.

#### *WHO Criteria*

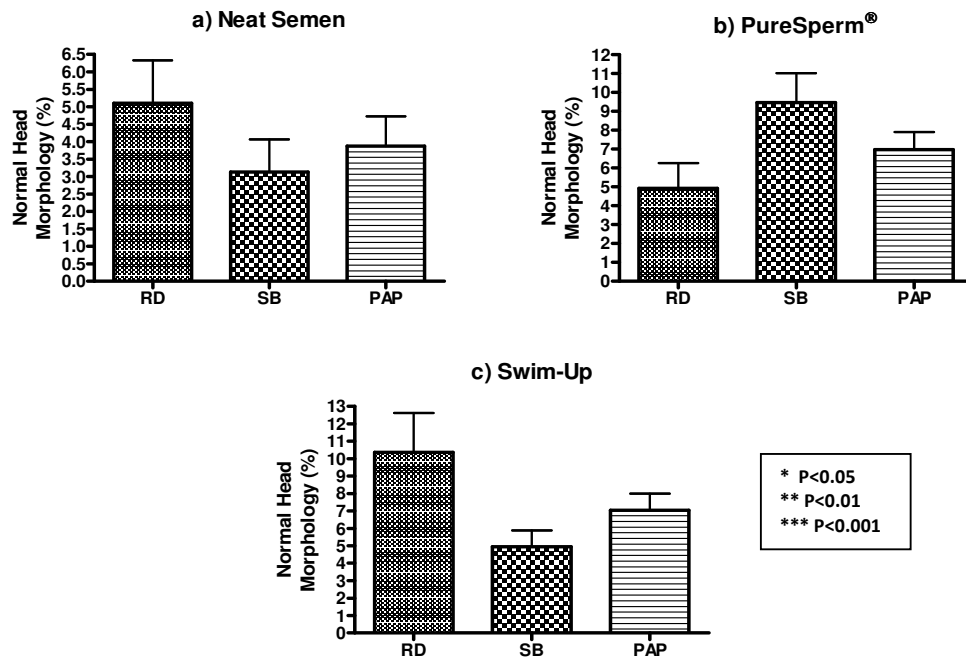
The analysis of normal morphological sperm forms according to WHO criteria for head morphology revealed no significant differences in the neat and swim-up fractions (See Fig. 17a & 17c). However, significance was found in the PureSperm<sup>®</sup> density gradient fraction, where RD-stained sperm was shown to differ significantly from those stained using SB (Fig. 17b: RD vs. SB:  $7.674\% \pm 1.727\%$  vs.  $23.87\% \pm 2.459\%$ ;  $p < 0.01$ ) and PAP (Fig. 17b: RD vs. PAP:  $7.674\% \pm 1.727\%$  vs.  $19.91\% \pm 2.295\%$ ;  $p < 0.05$ ).



**Figure 17a-c.** The effects of different morphology stains on sperm morphology analysis according to WHO criteria (n=20)

### *Tygerberg strict criteria*

The analysis of abnormal morphological sperm forms according to Tygerberg strict criteria for head morphology revealed no significant differences in the neat, PureSperm<sup>®</sup> density gradient and swim-up fractions (See Fig. 18a-c).



**Figure 18a-c.** The effects of different morphology stains on sperm morphology analysis according to Tygerberg strict criteria (n=20)

**Table VII:** The effects of different staining techniques on sperm head morphology in neat semen according to WHO and Tygerberg strict criteria (Mean  $\pm$  SEM) (n=20)

	W.H.O Criteria			Tygerberg Strict Criteria		
	Rapidiff®	SpermBlue®	Papanicolaou	Rapidiff®	SpermBlue®	Papanicolaou
Normal Head (%)	6.932 $\pm$ 1.410	10.290 $\pm$ 2.318	12.210 $\pm$ 1.983	5.105 $\pm$ 1.223	3.132 $\pm$ 0.932	3.874 $\pm$ 0.855
Abnormal Head (%)	93.068 $\pm$ 1.410	89.710 $\pm$ 2.318	87.790 $\pm$ 1.983	94.895 $\pm$ 1.223	96.868 $\pm$ 0.932	96.126 $\pm$ 0.855
Normal Sized Head (%)	37.440 $\pm$ 3.532	26.410 $\pm$ 2.796	29.320 $\pm$ 3.877	64.580 $\pm$ 4.077 <sup>aa</sup>	42.080 $\pm$ 3.803	33.520 $\pm$ 4.919 <sup>ccc</sup>
Micro Head (%)	19.390 $\pm$ 5.944 <sup>a</sup>	39.960 $\pm$ 5.429 <sup>b</sup>	62.30 $\pm$ 5.379 <sup>ccc</sup>	18.540 $\pm$ 4.670 <sup>aa</sup>	44.050 $\pm$ 5.276 <sup>b</sup>	64.360 $\pm$ 5.335 <sup>ccc</sup>
Macro Head (%)	43.140 $\pm$ 5.363	33.630 $\pm$ 5.230 <sup>bbb</sup>	8.137 $\pm$ 1.926 <sup>ccc</sup>	16.890 $\pm$ 2.937	13.870 $\pm$ 2.836 <sup>bb</sup>	2.126 $\pm$ 0.6429 <sup>ccc</sup>
Normal Shape Head (%)	51.920 $\pm$ 3.090 <sup>aaa</sup>	29.490 $\pm$ 4.167 <sup>bbb</sup>	62.860 $\pm$ 2.479	21.810 $\pm$ 2.551 <sup>a</sup>	11.160 $\pm$ 2.207 <sup>bbb</sup>	30.890 $\pm$ 2.769 <sup>c</sup>
Paintbrush Head (%)	1.911 $\pm$ 0.270	2.016 $\pm$ 0.370	1.826 $\pm$ 0.496	59.970 $\pm$ 3.458	49.540 $\pm$ 2.787	51.190 $\pm$ 3.415
Thin Head (%)	2.921 $\pm$ 0.782 <sup>aa</sup>	7.647 $\pm$ 1.208 <sup>bb</sup>	2.926 $\pm$ 0.648	7.995 $\pm$ 1.494 <sup>aaa</sup>	20.880 $\pm$ 2.835 <sup>bbb</sup>	6.121 $\pm$ 1.101
Round Head (%)	2.268 $\pm$ 0.661	1.405 $\pm$ 0.414 <sup>b</sup>	4.368 $\pm$ 1.106	1.079 $\pm$ 0.346	0.684 $\pm$ 0.254	2.553 $\pm$ 0.829
Tapering Head (%)	32.280 $\pm$ 2.827 <sup>aa</sup>	47.290 $\pm$ 4.184 <sup>bbb</sup>	16.470 $\pm$ 1.918 <sup>cc</sup>	2.705 $\pm$ 0.537 <sup>aa</sup>	8.358 $\pm$ 1.754 <sup>b</sup> bb	1.263 $\pm$ 0.4665
Amorphous Head (%)	8.742 $\pm$ 1.330	12.170 $\pm$ 1.179	11.550 $\pm$ 1.267	6.463 $\pm$ 1.397	9.395 $\pm$ 1.507	7.979 $\pm$ 1.065
Normal Acrosome (%)	29.400 $\pm$ 4.507 <sup>aaa</sup>	71.760 $\pm$ 3.633 <sup>bbb</sup>	38.620 $\pm$ 5.044	29.820 $\pm$ 4.045 <sup>aaa</sup>	71.760 $\pm$ 3.633 <sup>bbb</sup>	38.620 $\pm$ 5.044
Abnormal Acrosome (%)	70.910 $\pm$ 4.605 <sup>aaa</sup>	28.240 $\pm$ 3.633 <sup>bbb</sup>	61.380 $\pm$ 5.044	70.180 $\pm$ 4.045 <sup>aaa</sup>	28.240 $\pm$ 3.633 <sup>bbb</sup>	61.380 $\pm$ 5.044

<sup>a</sup> Rapidiff® vs. SpermBlue® P<0.05; <sup>aa</sup> Rapidiff® vs. SpermBlue® P<0.01; <sup>aaa</sup> Rapidiff® vs. SpermBlue® P<0.001

<sup>b</sup> SpermBlue® vs. Papanicolaou P<0.05; <sup>bb</sup> SpermBlue® vs. Papanicolaou P<0.01; <sup>bbb</sup> SpermBlue® vs. Papanicolaou P<0.001

<sup>c</sup> Papanicolaou vs. Rapidiff® P<0.05; <sup>cc</sup> Papanicolaou vs. Rapidiff® P<0.01; <sup>ccc</sup> Papanicolaou vs. Rapidiff® P<0.001



**Table VIII:** The effects of different staining techniques on head morphology in sperm obtained via the PureSperm<sup>®</sup> density gradient separation technique according to WHO and Tygerberg strict criteria (Mean ± SEM) (n=20)

	W.H.O Criteria			Tygerberg Strict Criteria		
	Rapidiff <sup>®</sup>	SpermBlue <sup>®</sup>	Papanicolaou	Rapidiff <sup>®</sup>	SpermBlue <sup>®</sup>	Papanicolaou
Normal Head (%)	14.120 ± 2.769 <sup>aa</sup>	28.410 ± 2.799	25.320 ± 2.819 <sup>c</sup>	11.090 ± 2.422	13.620 ± 2.069	11.890 ± 1.414
Abnormal Head (%)	85.880 ± 2.769 <sup>aa</sup>	71.590 ± 2.799	74.680 ± 2.819 <sup>c</sup>	88.910 ± 2.422	86.380 ± 2.069	88.110 ± 1.414
Normal Sized Head (%)	48.060 ± 3.083	43.660 ± 3.052	43.020 ± 2.980	64.930 ± 3.950	58.650 ± 4.406	49.670 ± 3.872 <sup>c</sup>
Micro Head (%)	25.300 ± 5.040	27.040 ± 4.519 <sup>b</sup>	46.240 ± 4.049 <sup>cc</sup>	26.780 ± 4.976	55.600 ± 28.17	47.740 ± 4.017
Macro Head (%)	26.650 ± 4.211	29.280 ± 3.580 <sup>bbb</sup>	10.730 ± 1.853 <sup>cc</sup>	8.300 ± 1.720	8.484 ± 1.342 <sup>bb</sup>	2.595 ± 0.6241 <sup>cc</sup>
Normal Shape Head (%)	68.260 ± 1.613 <sup>aaa</sup>	52.820 ± 3.168 <sup>bb</sup>	63.910 ± 2.540	32.840 ± 2.657 <sup>aa</sup>	20.160 ± 2.358	28.790 ± 2.513
Paintbrush Head (%)	2.232 ± 0.601	2.321 ± 0.438	2.868 ± 0.423	53.050 ± 3.351	59.610 ± 3.039	53.440 ± 3.203
Thin Head (%)	2.821 ± 0.708	5.816 ± 1.385	4.289 ± 0.821	4.537 ± 1.077 <sup>a</sup>	14.850 ± 4.533	6.974 ± 1.354
Round Head (%)	6.679 ± 1.525	3.832 ± 1.015	5.958 ± 1.294	3.205 ± 0.989	1.879 ± 0.585	2.805 ± 0.773
Tapering Head (%)	13.640 ± 1.567 <sup>aaa</sup>	25.740 ± 2.668 <sup>bb</sup>	14.260 ± 2.228	1.542 ± 0.386	4.395 ± 2.981	0.616 ± 0.184
Amorphous Head (%)	6.389 ± 0.759 <sup>a</sup>	9.479 ± 0.927	8.695 ± 0.939	4.837 ± 0.669	5.168 ± 1.767	7.389 ± 1.328
Normal Acrosome (%)	29.200 ± 5.474 <sup>aaa</sup>	75.440 ± 4.236 <sup>bbb</sup>	48.230 ± 5.071 <sup>c</sup>	29.200 ± 5.474 <sup>aaa</sup>	75.340 ± 4.217 <sup>bb</sup>	48.230 ± 5.071 <sup>c</sup>
Abnormal Acrosome (%)	70.800 ± 5.474 <sup>aaa</sup>	24.560 ± 4.236 <sup>bbb</sup>	51.770 ± 5.071 <sup>c</sup>	70.850 ± 5.487 <sup>aaa</sup>	24.660 ± 4.217 <sup>bb</sup>	51.770 ± 5.071 <sup>c</sup>

<sup>a</sup> Rapidiff<sup>®</sup> vs. SpermBlue<sup>®</sup> P<0.05; <sup>aa</sup> Rapidiff<sup>®</sup> vs. SpermBlue<sup>®</sup> P<0.01; <sup>aaa</sup> Rapidiff<sup>®</sup> vs. SpermBlue<sup>®</sup> P<0.001

<sup>b</sup> SpermBlue<sup>®</sup> vs. Papanicolaou P<0.05; <sup>bb</sup> SpermBlue<sup>®</sup> vs. Papanicolaou P<0.01; <sup>bbb</sup> SpermBlue<sup>®</sup> vs. Papanicolaou P<0.001

<sup>c</sup> Papanicolaou vs. Rapidiff<sup>®</sup> P<0.05; <sup>cc</sup> Papanicolaou vs. Rapidiff<sup>®</sup> P<0.01; <sup>ccc</sup> Papanicolaou vs. Rapidiff<sup>®</sup> P<0.001

**Table IX:** The effects of different staining techniques on head morphology in sperm obtained via the swim-up method according to WHO and Tygerberg strict criteria (Mean  $\pm$  SEM) (n=20)

	W.H.O Criteria			Tygerberg Strict Criteria		
	Rapidiff <sup>®</sup>	SpermBlue <sup>®</sup>	Papanicolaou	Rapidiff <sup>®</sup>	SpermBlue <sup>®</sup>	Papanicolaou
Normal Head (%)	16.830 $\pm$ 2.917	21.940 $\pm$ 2.218	25.140 $\pm$ 3.353	13.120 $\pm$ 2.531	10.950 $\pm$ 2.039	12.070 $\pm$ 1.701
Abnormal Head (%)	83.170 $\pm$ 2.917	78.060 $\pm$ 2.218	74.860 $\pm$ 3.353	86.880 $\pm$ 2.531	89.050 $\pm$ 2.039	87.930 $\pm$ 1.701
Normal Sized Head (%)	49.850 $\pm$ 3.317	43.820 $\pm$ 2.086	33.920 $\pm$ 4.423 <sup>cc</sup>	69.810 $\pm$ 3.635	70.010 $\pm$ 2.263 <sup>bbb</sup>	36.990 $\pm$ 4.991 <sup>ccc</sup>
Micro Head (%)	18.970 $\pm$ 4.516	13.710 $\pm$ 2.544 <sup>bbb</sup>	61.770 $\pm$ 5.187 <sup>ccc</sup>	20.890 $\pm$ 4.638	15.570 $\pm$ 2.679 <sup>bbb</sup>	62.360 $\pm$ 5.123 <sup>ccc</sup>
Macro Head (%)	31.170 $\pm$ 4.390	42.480 $\pm$ 3.948 <sup>bbb</sup>	4.312 $\pm$ 1.014 <sup>ccc</sup>	8.268 $\pm$ 1.772 <sup>aaa</sup>	24.060 $\pm$ 3.683 <sup>bbb</sup>	0.647 $\pm$ 0.242
Normal Shape Head (%)	66.930 $\pm$ 2.364 <sup>aaa</sup>	43.650 $\pm$ 3.212 <sup>bbb</sup>	70.160 $\pm$ 2.277	33.020 $\pm$ 3.270 <sup>aaa</sup>	14.750 $\pm$ 2.014 <sup>bbb</sup>	38.120 $\pm$ 2.564
Paintbrush Head (%)	2.963 $\pm$ 1.102	1.805 $\pm$ 0.344	1.959 $\pm$ 0.546	53.630 $\pm$ 3.674	51.270 $\pm$ 3.613	47.390 $\pm$ 3.155
Thin Head (%)	1.932 $\pm$ 0.625	2.305 $\pm$ 0.638	2.318 $\pm$ 0.820	3.805 $\pm$ 0.677 <sup>aaa</sup>	14.17 $\pm$ 2.592 <sup>bbb</sup>	3.988 $\pm$ 1.168
Round Head (%)	4.553 $\pm$ 1.354	2.079 $\pm$ 0.942	5.959 $\pm$ 1.400	2.116 $\pm$ 0.734	1.174 $\pm$ 0.651	2.712 $\pm$ 0.766
Tapering Head (%)	16.970 $\pm$ 2.014 <sup>aaa</sup>	40.240 $\pm$ 3.319 <sup>bbb</sup>	10.420 $\pm$ 1.842	1.268 $\pm$ 0.438 <sup>aa</sup>	9.232 $\pm$ 2.765 <sup>bb</sup>	0.5882 $\pm$ 0.211
Amorphous Head (%)	6.679 $\pm$ 0.882	9.926 $\pm$ 1.261	9.159 $\pm$ 0.981	4.584 $\pm$ 0.715 <sup>a</sup>	9.379 $\pm$ 1.570	7.206 $\pm$ 1.186
Normal Acrosome (%)	35.710 $\pm$ 5.416 <sup>aaa</sup>	70.460 $\pm$ 4.461	57.390 $\pm$ 6.116 <sup>c</sup>	33.130 $\pm$ 5.244 <sup>aaa</sup>	65.940 $\pm$ 5.622	57.390 $\pm$ 6.116 <sup>cc</sup>
Abnormal Acrosome (%)	64.290 $\pm$ 5.416	29.540 $\pm$ 4.461	42.610 $\pm$ 6.116 <sup>c</sup>	65.290 $\pm$ 5.410 <sup>aaa</sup>	34.060 $\pm$ 5.622	42.610 $\pm$ 6.116 <sup>cc</sup>
<sup>a</sup> Rapidiff <sup>®</sup> vs. SpermBlue <sup>®</sup> P<0.05; <sup>aa</sup> Rapidiff <sup>®</sup> vs. SpermBlue <sup>®</sup> P<0.01; <sup>aaa</sup> Rapidiff <sup>®</sup> vs. SpermBlue <sup>®</sup> P<0.001 <sup>b</sup> SpermBlue <sup>®</sup> vs. Papanicolaou P<0.05; <sup>bb</sup> SpermBlue <sup>®</sup> vs. Papanicolaou P<0.01; <sup>bbb</sup> SpermBlue <sup>®</sup> vs. Papanicolaou P<0.001 <sup>c</sup> Papanicolaou vs. Rapidiff <sup>®</sup> P<0.05; <sup>cc</sup> Papanicolaou vs. Rapidiff <sup>®</sup> P<0.01; <sup>ccc</sup> Papanicolaou vs. Rapidiff <sup>®</sup> P<0.001						

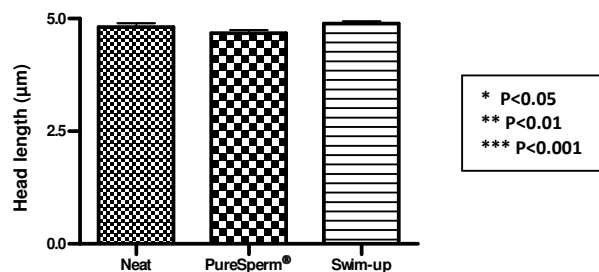
## 4.2 Comparison of two sperm separation techniques with regards to sperm morphometry, morphology, motility and concentration of the isolated population

To evaluate the difference of sperm separation methods, morphology, morphometry, motility parameters and concentration of sperm retrieved from two techniques were compared to each other as well as to those of sperm from neat unprocessed semen. Based on the results obtained in the first section of this study (*See section 4.1*), head morphology was analysed using the SpermBlue<sup>®</sup> staining technique. SpermBlue<sup>®</sup> was found to have the least impact on sperm head structure when compared to Rapidiff<sup>®</sup> and Papanicolaou stains.

### 4.2.1 Sperm head morphometry

#### *Head Length*

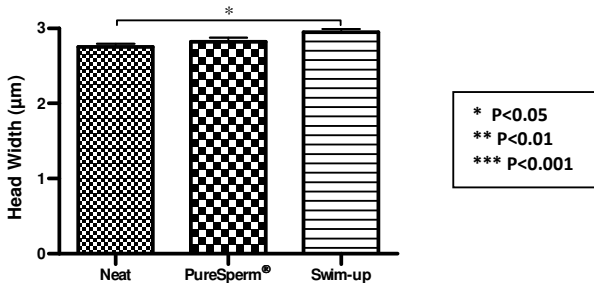
Results showed no significant differences in head length between the populations investigated (*See Fig. 19*).



**Figure 19.** Comparison of sperm head length from sperm in neat semen and those retrieved via two different semen preparation methods (n=20)

### Head Width

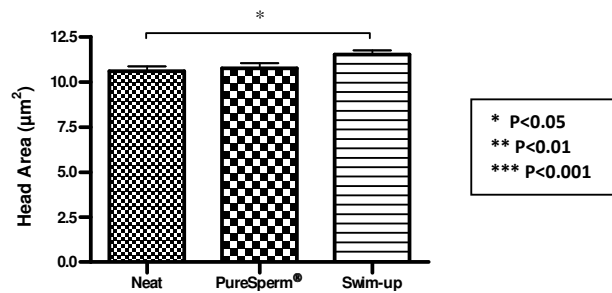
Investigation of sperm head width revealed that sperm retrieved via the swim-up method possessed a significantly greater mean head width than that observed in sperm of neat semen (Fig. 20: Swim-up vs. Neat:  $2.951\mu\text{m} \pm 0.04255\mu\text{m}$  vs.  $2.752\mu\text{m} \pm 0.04569\mu\text{m}$ ;  $p < 0.05$ ).



**Figure 20.** Comparison of sperm head width from sperm in neat semen and those retrieved via two different semen preparation methods (n=20)

### Head Area

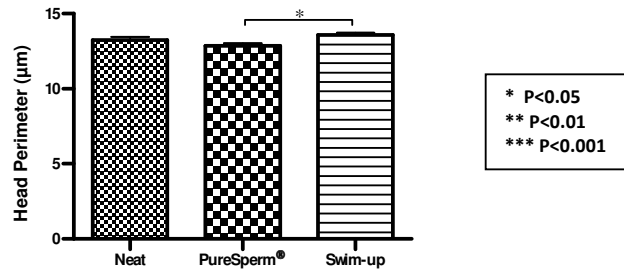
Significantly greater sperm head areas were observed in the swim-up fractions when compared to neat semen (Fig. 21: Swim-up vs. Neat:  $11.520\mu\text{m}^2 \pm 0.224\mu\text{m}^2$  vs.  $10.580\mu\text{m}^2 \pm 0.276\mu\text{m}^2$ ;  $p < 0.05$ ).



**Figure 21.** Comparison of sperm head area from sperm in neat semen and those retrieved via two different semen preparation methods (n=20)

### ***Head Perimeter***

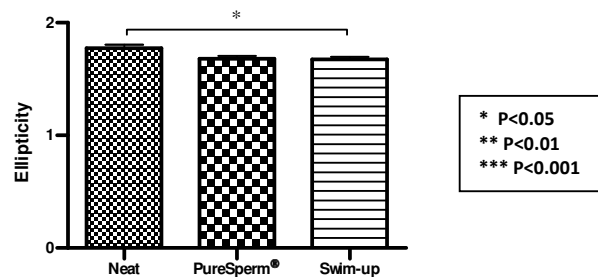
Results revealed that the swim-up fraction possessed a significantly greater mean sperm head perimeter than the PureSperm<sup>®</sup> density gradient fraction (Fig. 22: Swim-up vs. PureSperm<sup>®</sup> density gradient:  $13.580\mu\text{m} \pm 0.166\mu\text{m}$  vs.  $12.860\mu\text{m} \pm 0.167\mu\text{m}$ ;  $p < 0.01$ ).



**Figure 22.** Comparison of sperm head perimeter from sperm in neat semen and those retrieved via two different semen preparation methods (n=20)

### ***Head Ellipticity***

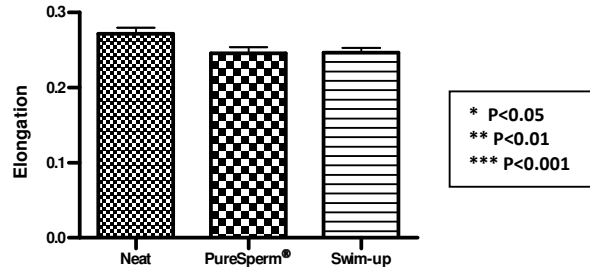
Head ellipticity in the swim-up was shown to be significantly lower than that of sperm in neat semen (Fig. 23: Neat vs. Swim-up:  $1.774 \pm 0.032$  vs.  $1.673 \pm 0.023$ ;  $p < 0.05$ ).



**Figure 23.** Comparison of sperm head ellipticity from sperm in neat semen and those retrieved via two different semen preparation methods (n=20)

### ***Head Elongation***

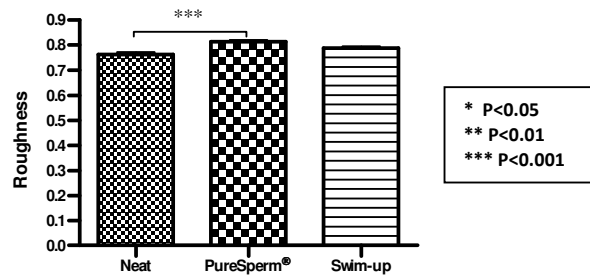
No significant differences in sperm head elongation were observed between the three populations compared (See Fig. 24).



**Figure 24.** Comparison of sperm elongation from sperm in neat semen and those retrieved via two different semen preparation methods (n=20)

### ***Head Roughness***

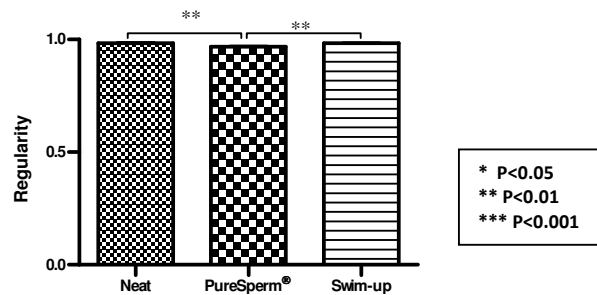
The PureSperm® density gradient fraction showed to have a significantly greater sperm head roughness than neat semen (Fig. 25: PureSperm® density gradient vs. Neat:  $0.8137 \pm 0.006$  vs.  $0.762 \pm 0.010$ ;  $p < 0.001$ )



**Figure 25.** Comparison of sperm head roughness from sperm in neat semen and those retrieved via two different semen preparation methods (n=20)

### Head Regularity

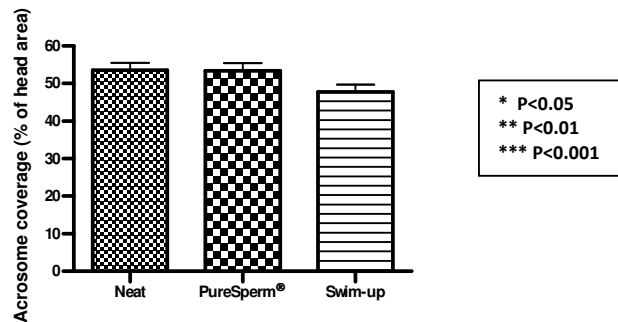
Sperm head regularity was significantly smaller in the PureSperm<sup>®</sup> density gradient fraction than that observed in the neat (Fig. 26: Neat vs. PureSperm<sup>®</sup> density gradient:  $0.984 \pm 0.004$  vs.  $0.968 \pm 0.003$ ;  $p < 0.01$ ) and swim-up fraction (Fig. 26: PureSperm<sup>®</sup> density gradient vs. Swim-up:  $0.968 \pm 0.003$  vs.  $0.984 \pm 0.002$ ;  $p < 0.01$ ).



**Figure 26.** Comparison of sperm head regularity from sperm in neat semen and those retrieved via two different semen preparation methods (n=20)

### Acrosome Coverage

No significant differences in acrosome coverage were found between the three groups (See Fig. 27).



**Figure 27.** Comparison of acrosome coverage from sperm in neat semen and those retrieved via two different semen preparation methods (n=20)

**Table X:** The comparison of head morphometry parameters of sperm obtained from different populations using SpermBlue<sup>®</sup> stain (Mean ± SEM) (n=20)

	<i>Neat</i>	<i>PureSperm<sup>®</sup></i>	<i>Swim-up</i>
Length (µm)	4.808 ± 0.085	4.681 ± 0.063	4.886 ± 0.056
Width (µm)	2.752 ± 0.046	2.825 ± 0.053	2.951 ± 0.043 <sup>c</sup>
Area (µm <sup>2</sup> )	10.580 ± 0.276	10.760 ± 0.272	11.520 ± 0.224 <sup>c</sup>
Perimeter (µm)	13.230 ± 0.226	12.860 ± 0.167 <sup>b</sup>	13.580 ± 0.166
Ellipticity	1.774 ± 0.032	1.679 ± 0.027	1.673 ± 0.023 <sup>c</sup>
Elongation	0.272 ± 0.008	0.246 ± 0.008	0.246 ± 0.007
Roughness	0.762 ± 0.010 <sup>aaa</sup>	0.814 ± 0.006	0.788 ± 0.007
Regularity	0.984 ± 0.004 <sup>aa</sup>	0.968 ± 0.003 <sup>bb</sup>	0.984 ± 0.002
Acrosome (%)	53.580 ± 2.005	53.350 ± 2.121	47.830 ± 1.848

<sup>a</sup> Neat vs. PureSperm<sup>®</sup> P<0.05; <sup>aa</sup> Neat vs. PureSperm<sup>®</sup> P<0.01; <sup>aaa</sup> Neat vs. PureSperm<sup>®</sup> P<0.001

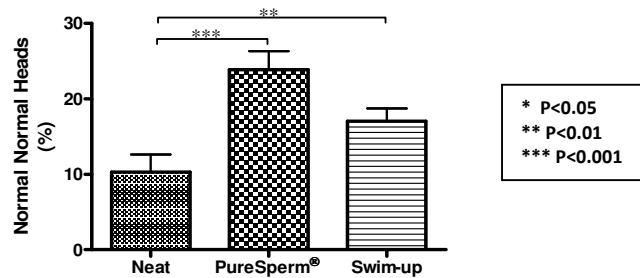
<sup>b</sup> PureSperm<sup>®</sup> vs. Swim-up P<0.05; <sup>bb</sup> PureSperm<sup>®</sup> vs. Swim-up P<0.01; <sup>bbb</sup> PureSperm<sup>®</sup> vs. Swim-up P<0.001

<sup>c</sup> Swim-up vs. Neat P<0.05; <sup>∞</sup> Swim-up vs. Neat P<0.01; <sup>∞∞</sup> Swim-up vs. Neat P<0.001

## 4.2.2 Sperm head morphology

### WHO Criteria

The swim-up (Fig. 28: Neat vs. Swim-up: 10.290 ± 2.318 vs. 17.040 ± 1.712; p<0.01) and PureSperm<sup>®</sup> density gradient (Fig. 28: Neat vs. PureSperm<sup>®</sup> density gradient: 10.290 ± 2.318 vs. 23.870 ± 2.459; p<0.001) populations both revealed a significantly higher percentages of sperm head normalities than the neat fraction.

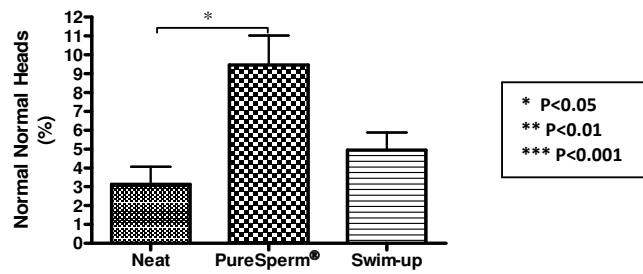


**Figure 28.** Comparison of abnormal sperm head morphology in different sperm subpopulations according to WHO criteria (n=20)



***Tygerberg strict criteria***

Morphology evaluation according to Tygerberg strict criteria revealed a significantly higher percentage of head normalities in the PureSperm<sup>®</sup> density gradient population than neat semen (Fig. 29: Neat vs. PureSperm<sup>®</sup> density gradient:  $3.132 \pm 0.932$  vs.  $9.468 \pm 1.553$ ;  $p < 0.05$ ).



**Figure 29.** Comparison of abnormal sperm head morphology in different sperm subpopulations according to Tygerberg strict criteria ( $n=20$ )

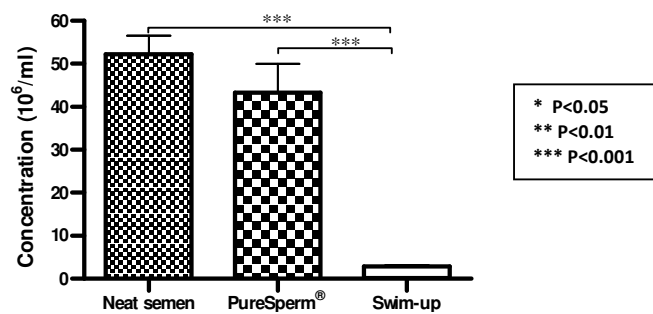
**Table XI:** The comparison of head morphology parameters according to WHO and Tygerberg strict criteria of sperm obtained from different populations using SpermBlue® (Mean ±SEM) (n=20)

	W.H.O Criteria			Tygerberg Strict Criteria		
	Neat	PureSperm®	Swim-up	Neat	PureSperm®	Swim-up
Normal Head (%)	10.290 ± 2.318 <sup>aaa</sup>	23.870 ± 2.459	17.040 ± 1.712 <sup>cc</sup>	3.132 ± 0.932 <sup>a</sup>	9.468 ± 1.553	4.953 ± 0.930
Abnormal Head (%)	89.710 ± 2.318 <sup>aaa</sup>	76.130 ± 2.459	82.960 ± 1.712 <sup>cc</sup>	96.868 ± 0.932 <sup>a</sup>	90.532 ± 1.553	95.047 ± 0.930
Normal Sized Head (%)	26.410 ± 2.796 <sup>aaa</sup>	43.660 ± 3.052	43.820 ± 2.086 <sup>ccc</sup>	42.080 ± 3.803 <sup>aa</sup>	58.650 ± 4.406	70.010 ± 2.263 <sup>ccc</sup>
Micro Head (%)	39.960 ± 5.429	27.040 ± 4.519	13.710 ± 2.544 <sup>ccc</sup>	44.05 ± 5.276	55.600 ± 28.17	15.570 ± 2.679
Macro Head (%)	33.630 ± 5.230	29.280 ± 3.580	42.480 ± 3.948	13.870 ± 2.836	8.484 ± 1.342 <sup>bbb</sup>	14.410 ± 2.128
Normal Shape Head (%)	29.490 ± 4.167 <sup>aaa</sup>	52.820 ± 3.168	43.650 ± 3.212 <sup>c</sup>	11.160 ± 2.207 <sup>a</sup>	20.160 ± 2.358	16.490 ± 2.356
Paintbrush Head (%)	2.016 ± 0.370	2.321 ± 0.438	1.805 ± 0.344	49.540 ± 2.787 <sup>a</sup>	59.610 ± 3.039	59.010 ± 2.730
Thin Head (%)	7.647 ± 1.208	5.816 ± 1.385	2.305 ± 0.638 <sup>cc</sup>	20.880 ± 2.835	14.850 ± 4.533	10.690 ± 1.636
Round Head (%)	1.405 ± 0.414	3.832 ± 1.015	2.079 ± 0.942	0.684 ± 0.254	1.879 ± 0.585	1.053 ± 0.690
Tapering Head (%)	47.290 ± 4.184 <sup>aaa</sup>	25.740 ± 2.668 <sup>b</sup>	40.240 ± 3.319	8.3580 ± 1.754	4.395 ± 2.981	5.468 ± 1.041
Amorphous Head (%)	12.170 ± 1.179	9.479 ± 0.9272	9.926 ± 1.261	9.395 ± 1.507	5.168 ± 1.767	7.284 ± 1.349
Normal Acrosome (%)	71.760 ± 3.633	75.440 ± 4.236	70.460 ± 4.461	71.760 ± 3.633	70.680 ± 5.716	70.410 ± 4.462
Abnormal Acrosome (%)	28.240 ± 3.633	24.560 ± 4.236	29.540 ± 4.461	28.240 ± 3.633	24.660 ± 4.217	29.590 ± 4.462
<sup>a</sup> Neat vs. PureSperm® P<0.05; <sup>aa</sup> Neat vs. PureSperm® P<0.01; <sup>aaa</sup> Neat vs. PureSperm® P<0.001 <sup>b</sup> PureSperm® vs. Swim-up P<0.05; <sup>bb</sup> PureSperm® vs. Swim-up P<0.01; <sup>bbb</sup> PureSperm® vs. Swim-up P<0.001 <sup>c</sup> Swim-up vs. Neat P<0.05; <sup>cc</sup> Swim-up vs. Neat P<0.01; <sup>ccc</sup> Swim-up vs. Neat P<0.001						

### 4.2.3 Sperm concentration and motility

#### *Sperm concentration*

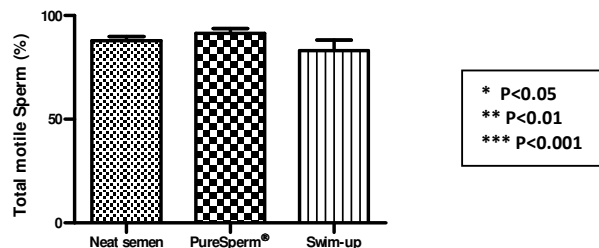
Sperm concentration analysis revealed that the swim-up fraction consistently yielded a significantly lower concentration than that of the neat (Fig. 30: Neat vs. Swim-up:  $52.210 \times 10^6/\text{ml} \pm 4.310 \times 10^6/\text{ml}$  vs.  $2.900 \times 10^6/\text{ml} \pm 0.258 \times 10^6/\text{ml}$ ;  $p < 0.001$ ) and PureSperm<sup>®</sup> density gradient (Fig. 30: PureSperm<sup>®</sup> density gradient vs. Swim-up:  $43.320 \times 10^6/\text{ml} \pm 6.609 \times 10^6/\text{ml}$  vs.  $2.900 \times 10^6/\text{ml} \pm 0.258 \times 10^6/\text{ml}$ ;  $p < 0.001$ ) fractions.



**Figure 30.** Comparison of sperm recovery following semen processing using two techniques (n=20)

#### *Total Motility (type a + b + c)*

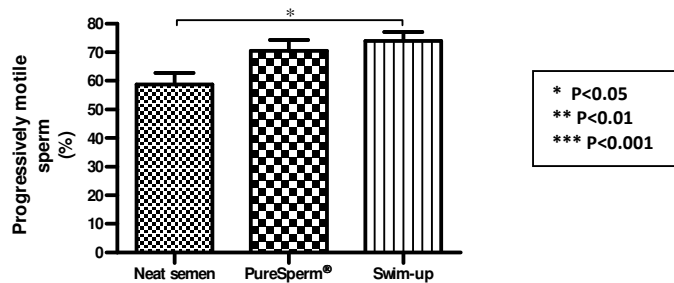
Total motility did not differ significantly between the groups (See Fig. 31).



**Figure 31.** Comparison of total motility in different sperm populations (n=20)

### ***Progressive Motility (type a + b)***

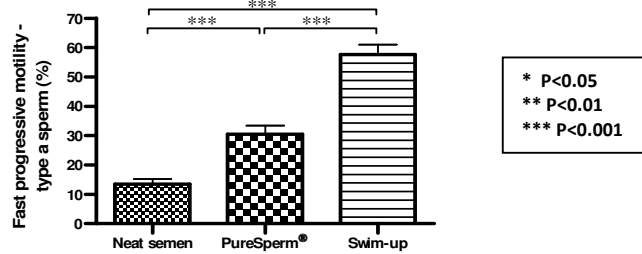
Results showed that progressive motility of the swim-up sperm was significantly greater than that of neat semen (Fig. 32: Swim-up vs. Neat:  $73.990\% \pm 3.101\%$  vs.  $58.680\% \pm 4.145\%$ ;  $p < 0.05$ ). Although the PureSperm<sup>®</sup> density gradient fraction appeared to be somewhat elevated in comparison to the neat fraction, no significance was found.



**Figure 32.** Comparison of progressive motility in different sperm populations ( $n=20$ )

### ***Fast Progressive (type a)***

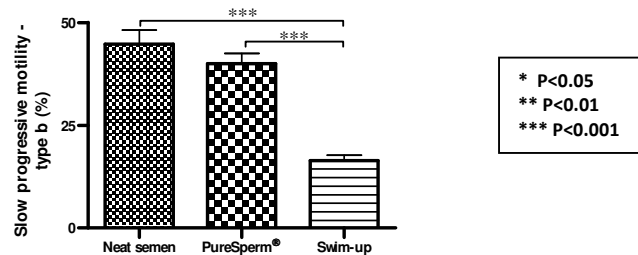
Results showed that the swim-up population had significantly higher concentrations of type a sperm than the PureSperm<sup>®</sup> density gradient fraction (Fig. 33: Swim-up vs. PureSperm<sup>®</sup> density gradient:  $57.590\% \pm 3.411\%$  vs.  $30.450\% \pm 2.966\%$ ;  $p < 0.001$ ) and neat semen (Fig. 33: Swim-up vs. Neat:  $57.590\% \pm 3.411\%$  vs.  $13.460\% \pm 1.740\%$ ;  $p < 0.001$ ). PureSperm<sup>®</sup> density gradient also had significantly larger concentrations of type a sperm than neat semen (Fig. 33: PureSperm<sup>®</sup> density gradient vs. Neat:  $30.450\% \pm 2.966\%$  vs.  $13.460\% \pm 1.740\%$ ;  $p < 0.001$ ).



**Figure 33.** Comparison of fast-progressive motility in different sperm populations (n=20)

### Slow-progressive (type b)

Neat semen and the PureSperm® density gradient fraction contained significantly higher concentrations of type b sperm than the swim-up (Fig. 34: Neat vs. Swim-up:  $44.740\% \pm 3.408\%$  vs.  $16.390\% \pm 1.329\%$ ;  $p < 0.001$  and PureSperm® density gradient vs. Swim-up:  $40.070\% \pm 2.442\%$  vs.  $13.460\% \pm 1.740\%$ ;  $p < 0.001$ ).

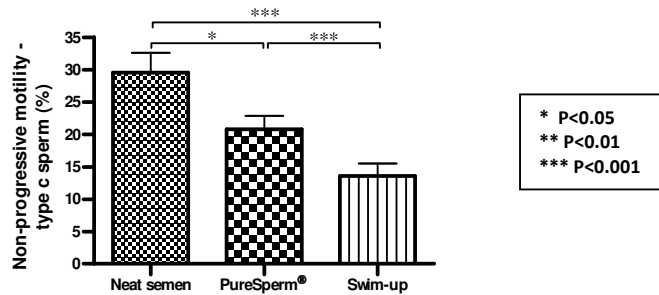


**Figure 34.** Comparison of slow-progressive motility in different sperm populations (n=20)

### Non-progressive (type c)

Neat semen contained significantly higher concentrations of type c sperm than the PureSperm® density gradient (Fig. 35: Neat vs. PureSperm® density gradient:  $29.590\% \pm 23.059\%$  vs.  $20.830\% \pm 2.021\%$ ;  $p < 0.05$ ) and swim-up (Fig. 35: Neat vs. Swim-up:  $29.590\% \pm 23.059\%$  vs.  $13.590\% \pm 1.947\%$ ;  $p < 0.001$ ) fractions respectively. Additionally, the PureSperm® density gradient fraction contained significantly larger concentrations of type c sperm than the swim-up fraction (Fig. 35:

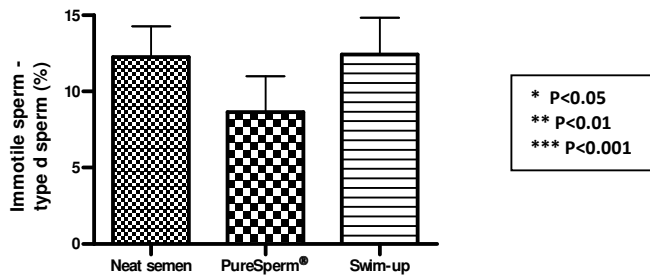
PureSperm<sup>®</sup> density gradient vs. Swim-up: 20.830% ± 2.021% vs. 13.590% ± 1.947%; p<0.001).



**Figure 35.** Comparison of non-progressive motility in different sperm populations (n=20)

***Immotile (type d)***

No significant differences were found between the different sperm populations and the concentration of type d sperm present.

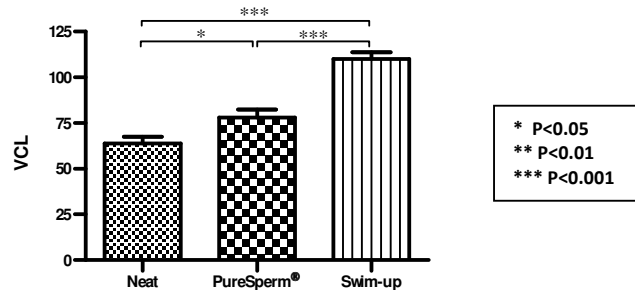


**Figure 36.** Comparison of immotile sperm in different sperm populations (n=20)

***VCL (Curvilinear speed)***

Results showed that curvilinear velocity (VCL) was highest in the swim-up fraction, followed by the PureSperm<sup>®</sup> density gradient and neat semen. The swim-up fraction had significantly higher values for VCL than the PureSperm<sup>®</sup> density gradient fraction (Fig. 37: Swim-up vs. PureSperm<sup>®</sup> density gradient: 110.100µm/s ±

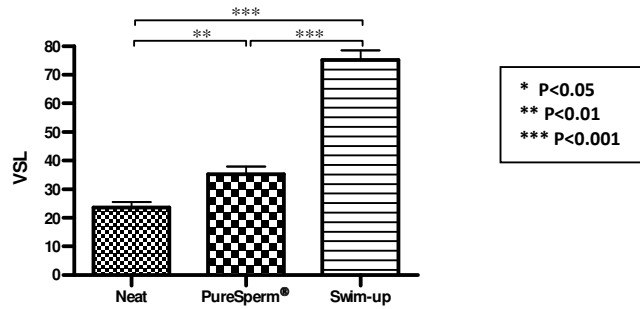
3.688 $\mu\text{m/s}$  vs. 78.120 $\mu\text{m/s} \pm 4.253\mu\text{m/s}$ ;  $p < 0.001$ ) and neat semen (Fig. 37: Swim-up vs. Neat: 110.100 $\mu\text{m/s} \pm 3.688\mu\text{m/s}$  vs. 63.780 $\mu\text{m/s} \pm 3.832\mu\text{m/s}$ ;  $p < 0.001$ ) respectively. Additionally, PureSperm<sup>®</sup> density gradient was shown to be significantly higher than the neat semen (Fig. 37: PureSperm<sup>®</sup> density gradient vs. Neat: 78.120 $\mu\text{m/s} \pm 4.253\mu\text{m/s}$  vs. 63.780 $\mu\text{m/s} \pm 3.832\mu\text{m/s}$ ;  $p < 0.05$ ).



**Figure 37.** Comparison of VCL in different sperm populations (n=20)

### ***VSL (Straight-line Velocity)***

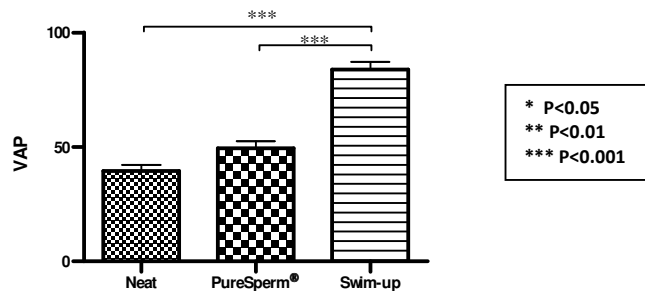
Results showed that straight-line velocity (VSL) was highest in the swim-up fraction, followed by the PureSperm<sup>®</sup> density gradient and neat semen. The swim-up fraction had significantly higher values for VSL than the PureSperm<sup>®</sup> density gradient fraction (Fig. 38: Swim-up vs. PureSperm<sup>®</sup> density gradient: 75.160 $\mu\text{m/s} \pm 3.430\mu\text{m/s}$  vs. 35.330 $\mu\text{m/s} \pm 2.655\mu\text{m/s}$ ;  $p < 0.001$ ) and neat semen (Fig. 38: Swim-up vs. Neat: 75.160 $\mu\text{m/s} \pm 3.430\mu\text{m/s}$  vs. 23.610 $\mu\text{m/s} \pm 1.853\mu\text{m/s}$ ;  $p < 0.001$ ) respectively. Additionally, PureSperm<sup>®</sup> density gradient was shown to be significantly higher than the neat semen (Fig. 38: PureSperm<sup>®</sup> density gradient vs. Neat: 35.330 $\mu\text{m/s} \pm 2.655\mu\text{m/s}$  vs. 23.610 $\mu\text{m/s} \pm 1.853\mu\text{m/s}$ ;  $p < 0.01$ ).



**Figure 38.** Comparison of VSL in different sperm populations (n=20)

### VAP (Average Path Velocity)

Results showed that average path velocity (VAP) was highest in the swim-up fraction, followed by the PureSperm® density gradient and neat semen. The swim-up fraction had significantly higher values for VAP than the PureSperm® density gradient fraction (Fig. 39: Swim-up vs. PureSperm® density gradient:  $83.760\mu\text{m/s} \pm 3.404\mu\text{m/s}$  vs.  $49.380\mu\text{m/s} \pm 3.013\mu\text{m/s}$ ;  $p < 0.001$ ) and neat semen (Fig. 39: Swim-up vs. Neat:  $83.760\mu\text{m/s} \pm 3.404\mu\text{m/s}$  vs.  $39.510\mu\text{m/s} \pm 2.593\mu\text{m/s}$ ;  $p < 0.001$ ) respectively. No significant differences were found between the neat semen and the PureSperm® density gradient fraction.

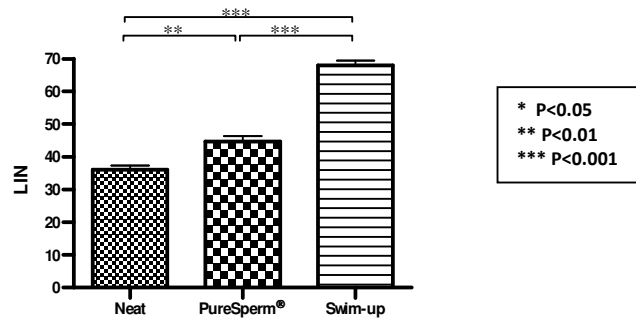


**Figure 39.** Comparison of VAP in different sperm populations (n=20)



### ***LIN (Linearity Index)***

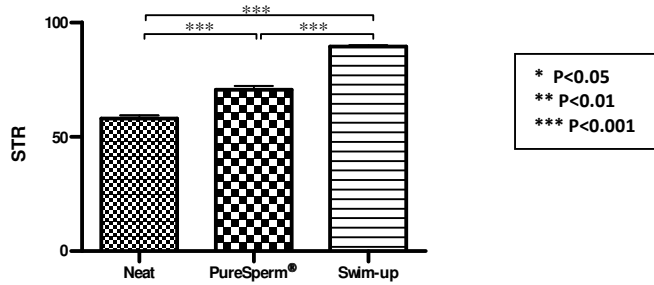
The linearity indexes (LIN) differed significantly between the three sperm populations (Fig. 40: Swim-up vs. PureSperm<sup>®</sup> density gradient:  $67.990 \pm 1.501$  vs.  $44.640 \pm 1.666$ ;  $p < 0.001$ ; PureSperm<sup>®</sup> density gradient vs. Neat:  $44.640 \pm 1.666$  vs.  $36.040 \pm 1.250$ ;  $p < 0.001$  and Swim-up vs. Neat:  $67.990 \pm 1.501$  vs.  $36.040 \pm 1.250$ ;  $p < 0.001$ ). The swim-up population possessed the highest LIN value, followed by the PureSperm<sup>®</sup> density gradient fraction and neat semen. The swim-up fraction differed significantly to the PureSperm<sup>®</sup> density gradient and neat semen.



**Figure 40.** Comparison of LIN in different sperm populations (n=20)

### ***STR (Straightness Index)***

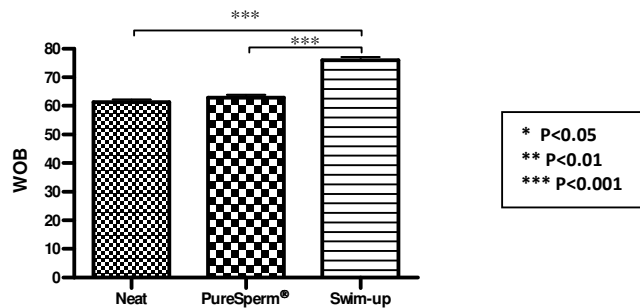
The straightness index (STR) differed significantly between the three sperm populations. The swim-up population possessed the highest STR value, followed by the PureSperm<sup>®</sup> density gradient fraction and neat semen. The swim-up fraction differed significantly to the PureSperm<sup>®</sup> density gradient (Fig. 41: Swim-up vs. PureSperm<sup>®</sup> density gradient:  $89.430 \pm 0.562$  vs.  $70.530 \pm 1.610$ ;  $p < 0.001$ ) and neat semen (Fig. 41: Swim-up vs. Neat:  $89.430 \pm 0.562$  vs.  $57.980 \pm 1.379$ ;  $p < 0.001$ ). Additionally, results showed that the STR of the PureSperm<sup>®</sup> density gradient fraction was significantly higher than that of the neat semen (Fig. 41: PureSperm<sup>®</sup> density gradient vs. Neat:  $70.530 \pm 1.610$  vs.  $57.980 \pm 1.379$ ;  $p < 0.001$ ).



**Figure 41.** Comparison of STR in different sperm populations (n=20)

**WOB (Oscillation Index)**

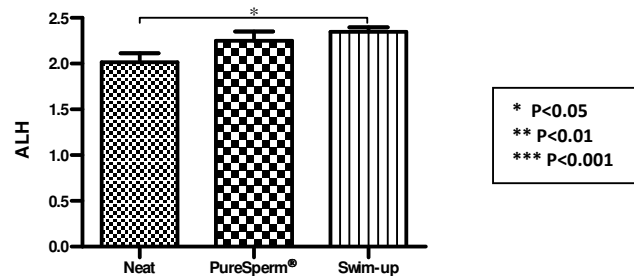
The WOB index of the swim-up fraction was significantly greater than that of both the neat semen (Fig. 42: Swim-up vs. Neat:  $75.910 \pm 1.285$  vs.  $61.360 \pm 0.965$ ;  $p < 0.001$ ) and PureSperm® density gradient fraction (Fig. 42: Swim-up vs. PureSperm® density gradient:  $75.910 \pm 1.285$  vs.  $62.920 \pm 1.069$ ;  $p < 0.001$ ).



**Figure 42.** Comparison of WOB in different sperm populations (n=20)

**ALH (Amplitude of Lateral Head Displacement)**

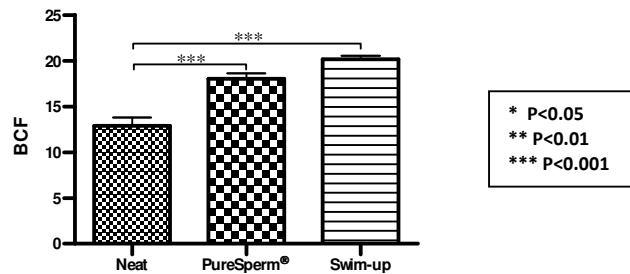
The swim-up fraction had an ALH significantly higher than that of neat semen (Fig. 43: Swim-up vs. Neat:  $2.350\mu\text{m} \pm 0.049\mu\text{m}$  vs.  $2.016\mu\text{m} \pm 0.098\mu\text{m}$ ;  $p < 0.05$ ).



**Figure 43.** Comparison of ALH in different sperm populations (n=20)

### ***BCF (Beat-cross Frequency)***

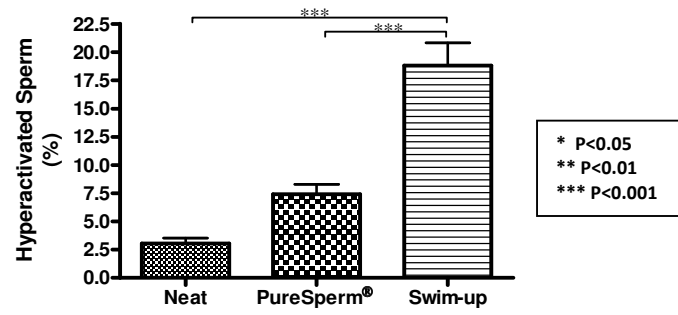
The beatcross frequency (BCF) of PureSperm® density gradient was significantly greater than that of neat semen (Fig. 44: PureSperm® density gradient vs. Neat: 18.030Hz ± 0.610Hz vs. 12.890Hz ± 0.907Hz; p<0.001) and swim-up populations (Swim-up vs. Neat: 20.170Hz ± 0.369Hz vs. 12.890Hz ± 0.907Hz; p<0.001).



**Figure 44.** Comparison of BCF in different sperm populations (n=20)

### ***Hyperactivated motility***

Hyperactivated motility, which is defined by ALH ( $3.75 \leq x \leq 15$ ), LIN ( $1 \leq x \leq 5$ ) and VCL ( $150 \leq x \leq 500$ ), was shown to differ significantly between neat semen and swim-up (Fig. 45: Swim-up vs. Neat®: 18.790 ± 2.018 vs. 3.042 ± 0.466; p<0.001) and PureSperm® density gradient and swim-up (Fig. : Swim-up vs. PureSperm® density gradient: 18.790 ± 2.018 vs. 7.421 ± 0.876; p<0.001).



*Figure 45: Comparison of hyperactivated motility in different sperm population (n=20)*

**Table XII:** The comparison of sperm concentration and motility parameters of sperm obtained from different populations (Mean  $\pm$ SEM) (n=20)

	<i>Neat Semen</i>	<i>PureSperm</i> <sup>®</sup>	<i>Swim-up</i>
Concentration (X10 <sup>3</sup> /ml)	52.210 $\pm$ 4.310	43.320 $\pm$ 6.009 <sup>bbb</sup>	2.900 $\pm$ 0.258 <sup>ccc</sup>
Total Motility (type a+b+c) (%)	87.790 $\pm$ 1.995	91.340 $\pm$ 2.345	82.970 $\pm$ 5.140
Progressive Motility (type a+b) (%)	58.680 $\pm$ 4.145	70.530 $\pm$ 3.765	73.990 $\pm$ 3.10 <sup>c</sup>
Non-progressive Motility (type c) (%)	29.590 $\pm$ 3.059 <sup>a</sup>	20.830 $\pm$ 2.021	13.590 $\pm$ 1.947 <sup>ccc</sup>
Static (%)	12.260 $\pm$ 1.994	8.647 $\pm$ 2.345	12.430 $\pm$ 2.408
Rapid (%)	56.910 $\pm$ 4.293	69.670 $\pm$ 3.829	65.250 $\pm$ 3.440
Medium (%)	19.840 $\pm$ 1.041	16.240 $\pm$ 1.556	13.080 $\pm$ 1.257 <sup>cc</sup>
Slow (%)	11.020 $\pm$ 3.345	5.421 $\pm$ 0.868	9.256 $\pm$ 1.936
Fast progressive (type a)	13.460 $\pm$ 1.740 <sup>aaa</sup>	30.450 $\pm$ 2.966 <sup>bbb</sup>	57.590 $\pm$ 3.411 <sup>ccc</sup>
Slow-progressive (type b)	44.740 $\pm$ 3.408	40.070 $\pm$ 2.442 <sup>bbb</sup>	16.390 $\pm$ 1.329 <sup>ccc</sup>
Non-progressive (type c)	29.590 $\pm$ 3.059 <sup>a</sup>	20.830 $\pm$ 2.021 <sup>bbb</sup>	13.590 $\pm$ 1.947 <sup>ccc</sup>
Immotile (type d)	12.260 $\pm$ 1.994	8.647 $\pm$ 2.345 <sup>bbb</sup>	12.430 $\pm$ 2.408
Circular tracks	69.890 $\pm$ 1.932 <sup>aa</sup>	58.500 $\pm$ 2.221 <sup>bbb</sup>	19.160 $\pm$ 1.617 <sup>ccc</sup>
VCL ( $\mu$ m/s)	63.780 $\pm$ 3.832 <sup>a</sup>	78.120 $\pm$ 4.253 <sup>bbb</sup>	110.100 $\pm$ 3.688 <sup>ccc</sup>
VSL ( $\mu$ m/s)	23.610 $\pm$ 1.853 <sup>aa</sup>	35.330 $\pm$ 2.655 <sup>bbb</sup>	75.160 $\pm$ 3.430 <sup>ccc</sup>
VAP ( $\mu$ m/s)	39.510 $\pm$ 2.593	49.380 $\pm$ 3.013 <sup>bbb</sup>	83.760 $\pm$ 3.404 <sup>ccc</sup>
LIN (%)	36.040 $\pm$ 1.250 <sup>aa</sup>	44.640 $\pm$ 1.666 <sup>bbb</sup>	67.990 $\pm$ 1.501 <sup>ccc</sup>
STR (%)	57.980 $\pm$ 1.379 <sup>aaa</sup>	70.530 $\pm$ 1.610 <sup>bbb</sup>	89.430 $\pm$ 0.562 <sup>ccc</sup>
WOB (%)	61.360 $\pm$ 0.965	62.920 $\pm$ 1.069 <sup>bbb</sup>	75.910 $\pm$ 1.285 <sup>ccc</sup>
ALH ( $\mu$ m/s)	2.016 $\pm$ 0.098	2.253 $\pm$ 0.101	2.350 $\pm$ 0.049 <sup>c</sup>
BCF (Hz)	12.890 $\pm$ 0.907 <sup>aaa</sup>	18.039 $\pm$ 0.610	20.170 $\pm$ 0.369 <sup>ccc</sup>
Hyperactivated sperm (%)	3.042 $\pm$ 0.466	7.421 $\pm$ 0.876 <sup>bbb</sup>	18.790 $\pm$ 2.018 <sup>ccc</sup>
Round Cells (%)	1.226 $\pm$ 0.496 <sup>a</sup>	0.078 $\pm$ 0.056	0.006 $\pm$ 0.006 <sup>cc</sup>
<sup>a</sup> Neat vs. PureSperm <sup>®</sup> P<0.05; <sup>aa</sup> Neat vs. PureSperm <sup>®</sup> P<0.01; <sup>aaa</sup> Neat vs. PureSperm <sup>®</sup> P<0.001 <sup>b</sup> PureSperm <sup>®</sup> vs. Swim-up P<0.05; <sup>bb</sup> PureSperm <sup>®</sup> vs. Swim-up P<0.01; <sup>bbb</sup> PureSperm <sup>®</sup> vs. Swim-up P<0.001 <sup>c</sup> Swim-up vs. Neat P<0.05; <sup>cc</sup> Swim-up vs. Neat P<0.01; <sup>ccc</sup> Swim-up vs. Neat P<0.001			

## CHAPTER 5

### DISCUSSION

#### **5.1 The influence of three different staining techniques on human sperm head morphometry and morphology**

Human sperm show great morphological dissimilarity, where a normal fertile ejaculate may contain sperm exhibiting considerable variations in both size and shape (36, 37, 79), with normal and pathological forms existing simultaneously. It has been shown by a number of investigators that definite correlations exist between normal morphology and fertilization rate *in vivo* and *in vitro* (66, 79). As a consequence, sperm morphology is generally accepted as the most useful parameter in the prediction of fertility (82). Despite the usefulness of this semen parameter, sperm morphology analyses have been confounded by a lack of standardization, particularly with regards to the staining techniques used for microscopic evaluation (37, 85).

Subsequent to sperm morphology evaluation emerging as an important clinical diagnostic tool over the past few decades, a number of differential staining techniques have been developed with the intention of providing effective, yet more rapid alternatives to the original time-consuming PAP staining technique (45). For this reason, various histological staining techniques used for light microscopic evaluation of morphology are currently employed in andrology laboratories and fertility clinics world-wide, each one differing in cost, technique and differential staining characteristics. Of the available techniques, only a few have been recognised by the WHO as being effective methods for routine semen analysis. These include the PAP, Shorr and DiffQuik staining methods (45, 64, 111). Despite this attempt by the WHO at gaining some level of standardization with regards to morphological staining techniques, there still remains a level of concern surrounding the effects these stains have on sperm morphometry. Since the osmolarities of the various solutions as well as the duration of the staining procedures differ considerably between most of the protocols, the possibility exists that they might differently influence sperm shape and size, and possibly final outcome of morphology evaluations (8).

The present investigation highlights these concerns by identifying the significant differences inherent in sperm morphometry and morphology evaluations following the use of two commonly used staining techniques, RD and PAP. In addition to these two stains, a new commercially available stain, SB, was investigated. The purpose of investigating the staining techniques with neat semen, as well as semen processed using the PureSperm<sup>®</sup> density gradient and swim-up methods, was to determine the repeatability and consistency of the characteristics of each stain. By doing this, the compatibility of the stains with different sperm populations (processed and unprocessed) could also be evaluated. The main aim of this study was to identify a staining technique which stains the sperm head differentially with minimal background staining, has little or no affect on sperm dimensions and is compatible with CASA.

### **5.1.1 Sperm head morphometry**

A comparative study by Gago et al. (1998), reported that sperm heads stained with RD displayed large morphometric parameters, whereas those stained with PAP displayed small morphometric parameters (37). These findings were comparable to those of the present study, where of the three stains investigated, RD-stained sperm were found to have the largest values for head length, width, area and perimeter, whereas sperm stained using PAP revealed the smallest mean values for these parameters in all three populations (neat semen, swim-up and PureSperm<sup>®</sup> density gradient) investigated. On the other hand, head width and area of sperm stained using SB were generally found to be intermediate to those of RD and PAP. However, no significant differences were found between RD and SB or between SB and PAP with regards to head length and head width respectively. From these results it is clear that different staining techniques do in fact cause various alterations to sperm head dimensions.

According to the WHO, sperm head morphometry is normal when head length falls between 4.0 – 5.0  $\mu\text{m}$  and head width falls between 2.5 – 3.5  $\mu\text{m}$  (52, 111). Head length and width measurements were found to be within the normal ranges for all of the stains investigated in the present study. Since the WHO-recommended morphometry values for normal sperm are based on 95% confidence intervals for

PAP-stained sperm (52), it is necessary to establish the morphometry of fresh, unstained sperm in order to more accurately determine the individual effects of each stain on sperm dimensions.

Although the current study did not include the evaluation of unstained semen, a study by Maree et al. 2010, involved a full morphometric analysis of fresh, unstained sperm from normozoospermic donors. Using the projection of a magnified image of the sperm onto a computer screen, Maree et al. were able to manually measure sperm head length and width using digital callipers. Their results revealed that unstained sperm had a mean head length and width of 4.79 ( $\pm$  0.27) and 2.82 ( $\pm$  0.19)  $\mu\text{m}$  respectively. These measurements, together with the results obtained in the present study suggest that RD causes sperm head swelling, while PAP causes shrinkage. For instance, the average head length obtained from the evaluation of neat semen was found to be 4.94 ( $\pm$ 0.09)  $\mu\text{m}$  and 4.19 ( $\pm$ 0.06)  $\mu\text{m}$  for RD and PAP respectively. Furthermore, the average head width for RD- and PAP-stained sperm was 3.03 ( $\pm$ 0.06) and 2.63 ( $\pm$ 0.04)  $\mu\text{m}$  respectively. In light of these results, it is apparent that a considerable amount of head shrinkage or swelling had occurred with the use of these two staining techniques. In contrast, SB displayed head width and length measurements of 4.81 ( $\pm$ 0.08)  $\mu\text{m}$  and 2.75 ( $\pm$ 0.05)  $\mu\text{m}$  respectively, comparable to those of unstained sperm obtained in the study by Maree et al. The number of macro and micro sperm heads further supports the view that RD causes head swelling and PAP causes shrinkage. In the neat semen population for instance, the mean percentage of micro heads were significantly greater in the PAP than in the RD-stained sperm. On the other hand, RD-stained sperm were shown to possess a significantly greater number of macro heads than those stained with PAP. The number of macro and micro heads in the SB-stained sperm were generally found to be intermediate to those present in RD- and PAP-stained sperm.

In order to more accurately assess the degree to which shrinkage or swelling occurred and whether these changes were uniform throughout the sperm head, ratios of head lengths as well as widths of the respective stains were calculated in relation to those of unstained sperm (*See Table XIII*). Ratios of [stained sperm : unstained sperm] higher than 1.00 indicated swelling, whereas those lower than 1.00 indicated shrinkage. A ratio of exactly 1.00 implied no change to the sperm dimensions had occurred. The



ratios calculated for head length were 1.03, 1.00 and 0.87 for RD, SB and PAP respectively. Similarly the ratios for head width were 1.07, 0.98 and 0.93 for RD, SB and PAP. Although these ratios once again confirm that RD caused sperm head swelling, it appears that this staining technique causes the sperm head to swell more in width than it does in length. Similarly, the ratios obtained for PAP revealed that shrinkage had occurred in both head length and width. However, it was apparent that the PAP staining technique had an increased tendency to cause shrinkage of the sperm head length-ways. The calculated ratios for SB suggested that no change had resulted with regards to head length, although a slight shrinkage in head width was apparent with this staining technique.

***Table XIII:** Ratios of head length and head width of unstained sperm, 4.79 and 2.82 respectively, to those stained using RD, SB and PAP. A ratio of 1.00 indicates no difference between the parameters and therefore implies zero shrinkage/swelling had occurred. Ratios higher than 1.00 indicate swelling, whereas ratios lower than 1.00 indicate shrinkage.*

	[RD : Unstained]	[SB : Unstained]	[PAP : Unstained]
<b>Ratio of head lengths</b>	1.03	1.00	0.87
<b>Ratio of head widths</b>	1.07	0.98	0.93

In view of aforementioned findings, it is evident that although a stain may cause shrinkage or swelling of the sperm head, these alterations in dimensions may not occur in a uniform fashion, where one staining technique may specifically cause a greater increase in head width and another may result in a greater decrease to head length. Collectively, these results suggest that SB has the least affect on sperm head dimensions when compared to the other two staining techniques. Based on the findings in this investigation, SB has been shown to give the most accurate indication of an unstained specimen.

In addition to the standard morphometric measurements of head length, width, area and perimeter, four SCA<sup>®</sup> software-calculated parameters include sperm head ellipticity, elongation, roughness and regularity. These factors take into account the standard sperm head measurements (width, length, area and perimeter), and give an indication of the relative shape of the head, allowing for the classification of sperm not only morphometrically, but also morphologically. For instance, a sperm cell may be classified as being abnormal due to having a head length or width which lies outside the normal ranges as specified by WHO criteria. From this information however, it is difficult to distinguish whether the sperm is round, tapered or normal in shape, for example. Hence, the four additional morphometric parameters were introduced by Microptic S.L. to indicate the relative morphology of a sperm cell using standard morphometric measurements. Ellipticity indicates the degree to which the sperm head is oval, thin or tapered. Similarly, elongation indicates the roundness of the head, roughness indicates regular-shaped or amorphous heads, and regularity indicates pyriform heads.

The present study revealed that sperm stained with SB had significantly greater head ellipticity, elongation and regularity than that of the other stains in the three populations investigated. Head roughness on the other hand was shown to be significantly less in the SB-stained sperm when compared to RD and PAP. These four morphometry parameters suggest SB-stained sperm heads are more thin, tapered and slightly more amorphous to those sperm stained using RD and PAP. Collectively, these findings were consistent with results obtained by the classification of sperm heads into various morphology groups, including tapered, round, amorphous and normal-shaped heads. As it has already been indicated, RD and PAP cause alterations to the sperm head in disproportionate fashions. Therefore, due to the finding that sperm stained with SB possess morphometric values closest to those of fresh, unstained sperm, it is believed that the values of SB for ellipticity, elongation, roughness and regularity are too reflective of the true morphology of the sperm population.

An additional morphometry parameter, which varied significantly between sperm stained with SB and the other two stains, is acrosome size. WHO guidelines stipulate that a normal acrosome coverage ranges from 40 to 70% of the total head area (111),

where anything less than 40% is considered abnormal. The size of an acrosome indicates the sperm's ability to undergo an acrosome reaction, a vital step in sperm-oocyte penetration at the site of fertilization. Consequently, an acrosome which is too small may compromise the sperm's ability to fertilize an oocyte. Likewise, the total absence of an acrosome implies a poor prognosis for fertilization as it has been shown in a number of studies that the percentage of sperm with normal, intact acrosomes is strongly linked to IVF success rates (18, 66). For the reasons just stated, acrosome size is a valuable parameter to assess for the prediction of male fertility (66).

The current investigation showed that in all three populations, the acrosome size of sperm stained with SB were significantly greater than those of RD and PAP. In the neat semen population for instance, sperm stained with SB displayed a mean acrosome coverage of 53.580 ( $\pm$  2.005) %, whereas PAP- and RD-stained sperm revealed acrosomes with a mean coverage of 32.580 ( $\pm$  1.616) % and 35.640 ( $\pm$  1.411) % respectively. Similar results were evident in the PureSperm<sup>®</sup> density gradient and swim-up populations, where acrosome coverage was also found to be significantly greater with sperm stained with SB than those stained with RD and PAP. These results indicate that according to WHO guidelines for sperm morphometry, only sperm stained using the SB staining technique reveal acrosomes with sizes which fall within normal ranges. Further supporting the notion that SB has the least effect on sperm dimensions and therefore best represents the morphology of a sample, the acrosome size of human sperm from normozoospermic donors has been calculated as possessing an average size of 54.6 ( $\pm$  3.22) % in relation to the sperm head (64). The measurements obtained by the manual evaluation of the acrosome of unstained sperm are comparable to the findings of acrosome size of SB-stained sperm, 53.580 ( $\pm$  2.005) % in the present study, indicating an accurate representation of the acrosome by SB.

It was established that on many occasions during the morphometric analyses of RD-stained sperm no clear, distinguishable boundary between the acrosomal and post-acrosomal regions existed. This caused the SCA<sup>®</sup> to inaccurately recognise the acrosome region. On a few occasions the acrosome failed to be recognized at all by the SCA<sup>®</sup>, resulting in the sperm being analysed as having no acrosome. On these occasions it was clear that RD failed to differentially stain the relevant components of

the sperm head; a vital factor required for morphology analysis. As a result, these incorrectly analysed sperm had to be eliminated from the morphometry evaluation which caused a significant increase in assessment time of the slides. Given that the evaluation of acrosome coverage can be done during a sperm morphometry assessment, it is imperative that the staining technique clearly differentiate the boundaries of the acrosome (64).

A further important characteristic to consider, particularly when investigating a morphology stain for use with CASA, is background staining, as this factor has the potential to negatively affect the detection of the sperm by the analysing software. An increased occurrence of background staining with RD was apparent particularly in the neat semen population, causing unclear boundaries, and sperm to be masked and analysed incorrectly. Similar findings were also reported in independent studies investigating RD (37, 106). In the current study, sperm heads were not adequately contrasted with the background, making it difficult for the SCA<sup>®</sup> to delineate the head boundary. As a result of this, RD-stained sperm were not easily detected during analysis. When however, the sperm was detected by the SCA<sup>®</sup>, it often resulted in an incorrect analysis of the sperm due to background particles being recognized as part of the sperm head. This once again increased the time of morphology analysis of RD slides, as a large number of sperm had to be eliminated due to background particles being included in the dimensions of the sperm head. RD-stained sperm in processed semen, namely the PureSperm<sup>®</sup> density gradient and Swim-up populations, had substantially less background staining, suggesting that one option to decrease this unfavourable characteristic would be to wash the semen prior to the preparation of smears and staining with RD. However this would undoubtedly increase preparation time and since most routine semen analyses are performed using unprocessed semen, this option may not be a practical one.

Overall, this investigation indicated that morphology staining techniques do result in significant variations to sperm head dimensions. These variations may be attributed to the differences in osmolarities between the semen and the respective fixatives and staining solutions, as well as the structure and positioning of the microfilaments contributing to the sperm head structure.

The cytoskeleton in the sperm head consists of the resistant structural proteins of the nucleus, and perinuclear theca, which are thought to be responsible, at least in part for shaping the nucleus and the more dynamic proteins of the cortical cytoskeleton of the sperm (33). According to Fouquet and Kann (1992), the exact distribution of actin filaments within the sperm head has been shown to vary considerably, possibly because of the methods of fixation, sensitivity of technique and membrane permeabilization during different staining techniques.

Osmolarity, which refers to the total concentration of active, ionized and unionized particles in a solution, has been calculated as approximately 360 mOsm/kg in human semen, but may range anywhere from 330 to 370 mOsm/kg (91). Since sperm are highly regionalized cells with localized membrane domains that have specific functions, they are known to act as good osmometers (64). The osmotic water permeability coefficient of human sperm membranes is very high while the associated activation energy is low, suggesting the presence of a porous membrane (1). When exposed to hypo-osmotic conditions, water enters the sperm in attempt to reach osmotic equilibrium. This inflow of water will increase sperm head volume and the plasma membrane will bulge, giving minimum surface to volume ratio and as a result, cause swelling (50). The opposite is true when placed in hyperosmotic conditions, which causes the sperm to lose water and subsequently shrink.

In support of this concept, the RD-fixative, RD-stain 1 and RD-stain 2 have been calculated as having osmolarities of 46, 182 and 170 mOsm/kg respectively (64), all of which are hypo-osmotic to semen. These large differences in relation to semen osmolarity could account for the level of head swelling encountered in this study with regards to the RD-staining technique. On the other hand, the PAP staining protocol involves the use of multiple solutions in a number of steps, making it difficult to identify where exactly in the process head shrinkage occurs. It may be speculated however, that a large degree of shrinkage can be attributed to the use of alcohol (ethanol) at many points in this technique, causing dehydration and shrinkage of the sperm head. Xylene, which is also used in the PAP staining technique, has also been attributed to sperm shrinkage as a result of hyperosmotic conditions (64).

Therefore, both the RD and PAP stain with their respective staining solutions may induce hypo- and hyper-osmotic conditions respectively in relation to semen, resulting in significant alterations to sperm head dimensions as was noted in the present investigation. Alternatively, SB solutions appear to have osmolarities very similar to that of human sperm. Maree et al. (2010), reported the two SB solutions as having osmolarities of 319 and 377 mOsm/kg respectively, with similar reports by van der Horst et al. (2009). In effect, these relatively isoosmotic solutions in relation to human semen are likely to have minimal influence on sperm head dimensions, due to nominal shrinkage or swelling. This can be confirmed by the results obtained in the present study, where sperm morphometry values for SB were found to be similar to the morphometric measurements of fresh, unstained sperm.

### **5.1.2 Sperm head morphology**

Although the results in the first part of this investigation have indicated that morphometric dimensions of the sperm head may be differentially altered depending on the staining method used, it still needs to be established whether or not these dissimilarities render significant variations in the overall morphology evaluation. In order to assess this, sperm head morphology was evaluated according to two commonly used evaluation criteria, WHO criteria and Tygerberg strict criteria.

Morphology evaluation according to WHO criteria did not reveal any significant differences between the stains in neat semen or the swim-up population. Surprisingly, in the PureSperm<sup>®</sup> density gradient population, significantly fewer normal sperm heads were observed with sperm stained with RD than the two remaining stains. This finding may be a consequence of the lack of consistency associated with the RD staining technique. Although the reason for this finding is not totally clear, it was evident that sperm in the PureSperm<sup>®</sup> density gradient morphology slides stained with RD were either stained too dark or too light. Because of this, it became difficult for the SCA<sup>®</sup> detection software to distinguish between the components of the sperm. Moreover, this may have resulted in the acrosome being analysed incorrectly, and sperm being classified as abnormal, therefore decreasing the number of normal forms present in the PureSperm<sup>®</sup> density gradient population of RD-stained sperm. This

inability of RD to stain sperm consistently suggests it is an unreliable stain to use for morphology evaluation.

No significant findings were observed between the stains when sperm morphology evaluation was performed using the stricter Tygerberg Criteria.

Although few significant differences were found between the morphology evaluations of the different stains, it should be addressed whether these findings are in fact clinically insignificant. For instance, no statistically significant difference was found between the RD-stained sperm mean normality (WHO criteria) of 6.932 % ( $\pm$  1.410) and PAP-stained sperm 12.210 % ( $\pm$  1.983) in the neat semen. Although a difference of approximately 5.278 % normal forms seems relatively small, it may become relevant when border-line cases are being investigated. According to the WHO, a semen sample is classified as normal for morphology when 30% or more sperm present as morphologically normal forms. With this in mind, it suggests a man with true sperm morphology that falls on this threshold of 30% could be classified as having abnormal or normal sperm morphology, purely based on the type of stain used during a semen evaluation. This fact should also be considered when a patient displays values fluctuating between the poor (P-group) and good (G-good) prognosis morphology groups with regards to Tygerberg strict criteria. It begs the question of whether in extreme cases these seemingly small, and statistically insignificant differences could result in inaccurate diagnoses and as a consequence lead to incorrect treatment.

In view of the increasing importance placed on sperm morphology evaluation results, it should be kept in mind that the values obtained with each staining method differ, albeit to small extents. Therefore, if sperm morphology evaluation cannot be standardised to a greater degree, a laboratory's normal values should at least be based on the specific staining method used in that laboratory (45). Ideally, stained sperm should have dimensions as close to sperm in fresh semen as possible, as was found with the SB staining method, resulting in accurate evaluations of sperm head morphometry.

## **5.2 Comparison of two sperm separation techniques with regards to sperm morphometry, morphology, motility and concentration of the isolated population**

Since the first successful IVF birth in the 1970's, the number of available semen preparation techniques has greatly expanded (46). Each technique with its own strengths and limitations, aims to isolate a population of high quality sperm which are free of seminal plasma and debris (17). Among the many techniques available, the swim-up and density gradient methods (i.e. PureSperm<sup>®</sup>) are considered most popular for use in the clinical setting (111). A large amount of research has been carried out on each technique, however there remains conflicting evidence as to which method is superior. As a consequence there remains no consensus regarding the best technique for isolating the desired sperm population for use in assisted reproduction (89). An ideal semen preparation technique would be one which isolates sperm with normal morphology, normal intact acrosomes, and high progressive motility (46), as these factors are known to optimise fertility success and birth rates with regards to ART (79).

In order to establish which of the swim-up or PureSperm<sup>®</sup> density gradient technique provides optimal results, this study investigated the outcome of each method by evaluating the morphometry, morphology, motility and concentration of sperm in the isolated populations. Based on the results obtained in the first part of this research project, sperm morphology smears were stained using the SB morphology staining technique, as it is likely to provide the most accurate indication of the true morphometry and morphology of the given sperm population.

### **5.2.1 Sperm head morphometry**

Morphometry results in this study showed that there were no significant differences in sperm head length between the groups. However, head width and area were found to be significantly greater in sperm obtained via the swim-up method than those in the neat semen samples. Similarly, a study by Henkel et al. (2003), showed that sperm obtained following a swim-up had significantly larger head areas and widths when compared to those of neat semen (8). It was also evident that sperm from the swim-up



population had significantly greater head perimeters than those from the PureSperm<sup>®</sup> density gradient population. Collectively, these morphometry results suggest that the swim-up method has the tendency to isolate sperm with slightly larger heads.

Further assessment of morphometry parameters indicated that the swim-up method yielded sperm with a significantly lower ellipticity index than sperm in the neat semen fraction. This may be explained by the findings in the previous section, where the swim-up sperm were shown to have significantly greater head widths than those of the neat semen. Since ellipticity is expressed as a ratio of head length to head width, an increase in head width will directly result in a decrease ellipticity, and vice versa. Higher ellipticity indexes indicate the presence of more oval-shaped sperm heads, where lower indexes indicate thinner sperm heads. In accordance to this, sperm obtained from the swim-up method appear to have heads which are more oval in shape. To explain this finding, a study by Gage (1998), suggested that the size and shape of mammalian sperm heads are under hydrodynamic selection for optimal swimming efficiency, and therefore more slender, oval-shaped sperm will result following a swim-up. In further support of this, the morphologic classification of sperm heads into groups revealed that sperm obtained via the swim-up method were found to have significantly less thin heads and significantly more normal-shaped (viz. oval) heads when compared to the neat semen population.

Furthermore, sperm obtained via the PureSperm<sup>®</sup> density gradient method were shown to possess significantly higher indexes for roughness and regularity than those sperm in the neat semen and swim-up populations. Head regularity indicates the degree to which the sperm head is pyriform or symmetrical in shape, where lower values indicate more symmetrical sperm heads. Roughness on the other hand indicates amorphism or irregularity of the sperm head. These findings therefore imply that the PureSperm<sup>®</sup> density gradient separation technique has the tendency to select for more regular, symmetrical sperm heads.

Although both the PureSperm<sup>®</sup> density gradient and swim-up separation techniques isolated populations of sperm whose acrosome sizes fell within normal ranges as advised by the WHO, no significant differences were evident between the groups with regards to acrosome size. These findings therefore indicate that neither of the two

semen preparation techniques investigated select for sperm with larger acrosomes as previous studies may have suggested .

Collectively, the results obtained by means of sperm morphometry evaluation of the three different populations (neat semen, swim-up and PureSperm<sup>®</sup> density gradient) in this study have suggest that sperm undergo morphometric selection, which may be different depending on the sperm preparation technique employed. For instance, where sperm separated by the swim-up may undergo hydrodynamic selection for improved motility, those separated by the PureSperm<sup>®</sup> density gradient method are selected according to density, which perhaps in some way may be associated with certain morphometric characteristics.

### **5.2.2 Sperm head morphology**

It has been shown time and again, the strong link which exists between sperm morphology and fertility success rates, both *in vitro* and *in vivo*. Incidentally, sperm head defects have been suggested as markers for other sperm defects that drastically impair fertility (79). Based on this theory, one of the primary aims of sperm separation is to optimize the normal morphology of the resultant population.

The morphometric evaluation and comparison of sperm obtained via the PureSperm<sup>®</sup> density gradient and swim-up methods in the previous section, showed a number of significant differences in sperm head dimensions when compared to those of the neat semen samples. However, owing to the importance of sperm morphology in the clinical setting, it is necessary to determine whether these differences in morphometry translate into significant improvements of morphology following the application of sperm separation techniques.

Morphology evaluations of the different populations, when assessed according to WHO criteria, showed that both the PureSperm<sup>®</sup> density gradient and swim-up methods yielded significantly higher percentages of sperm with morphologically normal heads than those in neat semen. This finding confirms that both methods are indeed successful at extracting morphologically normal sperm from a neat semen sample possessing heterozygous forms.

Morphology evaluation according to Tygerberg strict criteria, revealed a significant improvement in the percentage of morphology in the PureSperm<sup>®</sup> density gradient population than in the neat semen samples. Although sperm from the swim-up population appeared to have a noteworthy improvement in normal morphology according to Tygerberg strict criteria when compared to neat semen, this difference was found to be statistically insignificant.

Taking into consideration the results obtained through the morphological evaluation according to both WHO and Tygerberg strict criteria, it is apparent that the sperm obtained via the swim-up method contained more border-line forms than the PureSperm<sup>®</sup> density gradient population. Border-line morphology, when analysed according WHO criteria, are considered morphologically normal. On the other hand, border-line forms when analysed according to Tygerberg strict criteria, are regarded as abnormal. This main distinguishing factor between the two morphology criteria, may explain the significant difference observed between normal morphology of sperm in the neat semen and swim-up when analysed according to WHO criteria, but not Tygerberg strict criteria.

Collectively, the results obtained from the morphology evaluation of the three different sperm populations confirm that both semen preparation techniques, namely PureSperm<sup>®</sup> density gradient and swim-up, do indeed select for sperm with improved morphology. It is well established that Tygerberg strict criteria are more predictive than WHO criteria with regards to IVF success (110, 111). This suggests that the PureSperm<sup>®</sup> density gradient technique is superior to the swim-up method in isolating sperm with normal morphology. Because of this, PureSperm<sup>®</sup> density gradient may be a more favourable choice of sperm separation technique for use in a clinical setting where a higher quality of sperm are required.

### **5.2.3 Sperm concentration and motility**

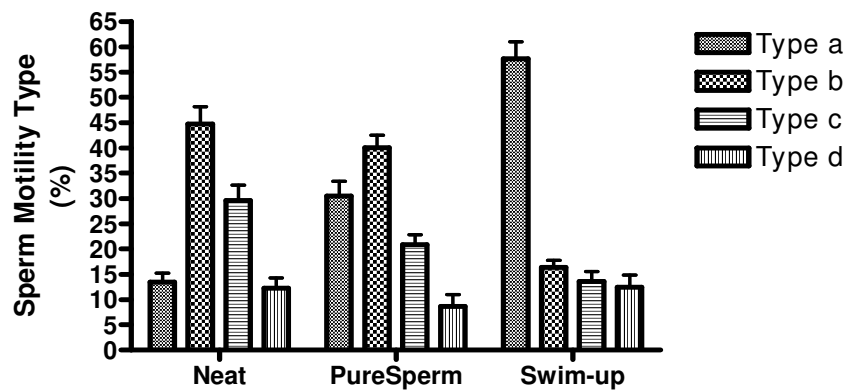
Evaluation of the functional capacity of human sperm *in vitro* is likely to be of great value in the assessment of a man's fertility potential (48). Although it has been established that sperm morphology is a very useful tool in the prediction of artificial reproductive success, in some cases sperm motility is thought to be just as important

(16). This is particularly found to be the case when IUI and IVF are concerned, where the ability of the sperm to actively migrate towards the oocyte is of utmost importance (31, 104). According to Mortimer (1994), the success or applicability of a sperm-washing method can be considered in terms of the yield of motile sperm that one obtains at the end of the technique. For this reason, it is important that along with sperm morphology, motility is also considered when applying sperm washing procedures (101).

Traditionally, total sperm motility and total progressive motility are viewed as two of the most important indicators of sperm functional integrity. However, since the introduction of various CASA systems, the analysis of sperm motility has allowed for the simultaneous evaluation of sperm velocity parameters, which in recent years have proved to be useful in the prediction of fertility success rates.

This investigation showed that the swim-up method resulted in the isolation of a significantly higher percentage of progressively motile sperm than was present in the neat semen population. However, no significant improvement in the percentage of progressively motile sperm was evident in the PureSperm<sup>®</sup> density gradient population. Progressive motility is expressed as the sum of the percentage of *type a* and *b* sperm, which are fast and slow progressively motile sperm respectively. The percentage of *type a* sperm were significantly higher in the swim-up and PureSperm<sup>®</sup> density gradient population than in the neat semen. Furthermore, the percentage of *type a* sperm in the swim-up population was found to be significantly greater than that of the PureSperm<sup>®</sup> density gradient population. However, the percentage of *type b* sperm were significantly lower in the swim-up fraction when compared to the other populations. The percentage of *type c* sperm were also significantly reduced following the swim-up and PureSperm<sup>®</sup> density gradient. Collectively, these results suggest that both the swim-up and PureSperm<sup>®</sup> density gradient have a stronger selection for fast progressively motile (*type a*) sperm, while the swim-up has a decreased tendency to isolate *type b* sperm (See Figure 46). Surprisingly, although the PureSperm<sup>®</sup> density gradient was effective at reducing the percentage of *type d* (immotile) sperm, the swim-up showed no significant decrease in the isolated population. The reason for this is unclear, as it is expected during a swim-up that only those sperm possessing adequate motility would be able to break free of the seminal plasma and enter the

culture media. However, it may be speculated that this unusual finding is a result of a methodological factor. It must be considered that with the aspiration of the supernatant containing the motile fraction of sperm following a swim-up, perhaps not enough care was taken to avoid sampling beneath the interface between the semen and the culture media. Considering this possibility, semen (containing sperm of all grades of motility) may have been incorporated with the culture media containing the “swim-up sperm” which was then analysed for motility. Perhaps to some degree, this may account of the unexpected percentage of immotile sperm observed in the swim-up population.



**Figure 46:** Visual comparison of the distribution of overall motility in the different sperm populations

Surprisingly, total motility of the isolated sperm populations remained unchanged following the application of the two sperm separation techniques. It may be hypothesized that the measurement of total motility in the unprocessed semen and PureSperm<sup>®</sup> density gradient fraction was somewhat affected by the concentration of the sperm in these populations. The possibility exists that a high concentration of sperm may lead to an increased incidence of collisions between sperm, causing immotile sperm to be recognized as being motile. Baring this in mind, motility evaluations may have resulted in a decreased percentage of *type d* (static) sperm. If

this indeed occurred in the present study, due to their high sperm concentrations it is expected that the unprocessed semen and PureSperm<sup>®</sup> fractions displayed a higher total motility than what was actually present. Due to the very low concentration of sperm in the swim-up fraction, it is unlikely that collisions between sperm would be of great significance. This theory is based on speculation and therefore requires further investigation. For future studies, it should be suggested that all sperm concentrations be adjusted and standardized prior to motility evaluation.

While progressive motility is required for the penetration of cervical mucus, another type of motility, known as hyperactivated motility, is required at the site of fertilization (44). Hyperactivation, which is defined as a vigorous type of movement observed during sperm capacitation, is required for zona pellucida penetration, and has been shown to be predictive of fertility success *in vitro* (28). Hyperactivated sperm have been positively correlated with fertility success rates with treatments such as IUI, GIFT and IVF (87). The functional significance of hyperactivation may be related to the increased mechanical forces generated by the high amplitude flagellar beat at the penetration site on or in the zona pellucida (16, 28, 52, 90). *In vivo*, hyperactivation is initiated as the sperm leave the seminal plasma and encounter capacitation-inducing factors present within the female reproductive tract (90). Therefore, semen preparation *in vitro* should ensure that sperm are adequately removed from the seminal plasma and placed in a culture media containing relevant capacitation stimulants to enable the sperm to enter a hyperactivated state.

Using CASA systems, the percentage of hyperactivated motility in a population may be evaluated in accordance to ALH, LIN and VCL measurements. In the present study, hyperactivated sperm were recognised as those having an ALH of  $3.75 \leq x \leq 15 \mu\text{m}$ , a LIN of  $1 \leq x \leq 5$  and a VCL of  $150 \leq x \leq 500 \mu\text{m/s}$ . Results showed that the percentage of hyperactive sperm were significantly greater following the swim-up method when compared to the sperm of the neat semen and PureSperm<sup>®</sup> density gradient populations, while the PureSperm<sup>®</sup> density gradient separation technique showed no significant improvement in the percentage of hyperactivated sperm. A possible reason for this may be attributed to the differences in chemical compositions of the media used for performing the PureSperm<sup>®</sup> density gradient centrifugation and

the sperm swim-up. Although, this theory is based on mere speculation and requires further investigation.

The added advantage CASA in the assessment of sperm motility is the measurement of velocity or kinematic motility parameters. There is adequate evidence to suggest that single or combinations of motility kinematic measurements are related to several important aspects of sperm function, which include penetration of cervical mucus, *in vitro* fertilization of oocytes and unassisted conception *in vivo* (35, 48, 60, 65, 83). In previous studies, VAP, VSL, VCL, STR, BCF and ALH have all individually shown a significant positive correlation with pregnancy rates *in vitro*, although to varying degrees (6, 31, 48, 49, 88, 101). For instance, VAP and ALH have been identified as parameters which best indicate a sperm cell's ability to penetrate cervical mucus (75). Results in the present study showed significant increases in VCL, VSL, LIN, STR and BCF in the swim-up and PureSperm<sup>®</sup> density gradient populations in comparison to unprocessed semen. However, all investigated kinematic values obtained from sperm in the swim-up population were significantly greater than those obtained from the PureSperm<sup>®</sup> density gradient fraction. Additionally, VAP, WOB and ALH of sperm in the swim-up population were also found to have significantly greater values than those of the neat semen population.

According to Mortimer et al. (2000), an important fundamental concept, when considering the diagnostic implication of sperm movement, is that sperm do not exhibit a single pattern of motility throughout their lifespan. Rather, their motility patterns change in accordance to their physiological needs. As a result, a greater understanding of how particular characteristics of sperm motion can relate to specific sperm functions is essential to realizing the potential inherent in the objective measurement of sperm movement characteristics. Hence, although improved velocity parameters have been repeatedly linked to fertility success rates, more emphasis needs to be placed on attaining clearer reference values which may be useful in understanding their respective clinical significance.

An additional factor to consider when investigating the effectiveness of a semen preparation technique is its ability to remove the desired population of sperm from the seminal plasma and harmful substances which may produce excessive ROS. When working with semen *in vitro*, it is strongly recommended that the sperm be removed

from the seminal plasma as soon as possible, as exposure to harmful factors can be highly detrimental to sperm functional integrity. Round cells, which are recognized as lacking the typical characteristics of sperm (51), are measured by the SCA<sup>®</sup> during motility analysis. Round cells may be either spermatogenic or non-spermatogenic in origin, and are often classed together. Since round cells include the presence of leukocytes which are known to generate excessive Reactive Oxygen Species (ROS) levels, high concentrations in the semen are assumed to be linked to subfertility, or at least impaired fertility. Results showed that both the PureSperm<sup>®</sup> density gradient and swim-up methods were equally successful at removing a large percentage of round cells from the neat semen, indicating that both of these methods are effective forms of sperm washing.

Collectively, the evaluation of motility parameters following the two semen preparation techniques have indicated that although a number of improvements exist following the PureSperm<sup>®</sup> density gradient method, the swim-up method yields superior results with regards to motility. However, these favourable results are associated with a considerable drawback, inherent in the sperm yield following a swim-up. In this study, the swim-up population possessed an average sperm concentration of  $2.900 (\pm 0.258) \times 10^6$ . The PureSperm<sup>®</sup> density gradient method on the other hand, isolated a population of sperm with a mean concentration of  $43.320 (\pm 6.009) \times 10^6$ . Numerous independent studies have reported the unfavourably low sperm recovery rate following a sperm swim-up (46, 88), which may become particularly problematic in cases where a high quantity of sperm are required for fertility treatment.

With the intention of improving sperm yield, it is suggested that the duration of the swim-up be increased from 20 to 60 minutes. Periods of longer than 60 minutes would not be advised due to factors associated with the increased exposure of the sperm to ROS, which may become detrimental to sperm. Alternatively, a swim-up may be performed from a washed sperm pellet as an alternative to the swim-up method employed in this study using a direct swim-up from unprocessed semen.



#### **5.2.4 Considerations when selecting a sperm separation technique**

This study showed that while the PureSperm<sup>®</sup> density gradient method was superior at isolating sperm with normal morphology, the swim-up method proved to be more effective at isolating sperm with superior motility. Based on these results, the lack of consensus surrounding the most appropriate sperm separation technique for use in fertility clinics becomes understandable. There remains insufficient evidence to recommend any specific preparation technique and therefore the selected method of semen preparation should be viewed in terms of the requirements of the specific fertility treatment (i.e. IVF, IUI, GIFT, ICSI) as well as the specific characteristics of the semen sample to be processed. With fertility treatments such as ICSI which require low concentrations of sperm with high motility, the swim-up preparation technique may be appropriate. However, the PureSperm<sup>®</sup> density gradient technique may be a more suitable method of choice in the case of IVF and IUI, where higher concentrations of sperm are required.

In addition to the type of fertility treatment, it is necessary that the quality of the semen sample for processing is considered prior to choosing an appropriate sperm preparation method. According to Henkel et al. (2003), the swim-up separation method may prove to be problematic and time-consuming in the case of a sample with poor motility (4), and it should therefore be abandoned in favour of an alternative sperm preparation technique such as PureSperm<sup>®</sup> density gradient. Similarly, performing a swim-up with highly viscous semen would prove to be equally problematic. It may be suggested that the swim-up separation technique be restricted to ejaculates with high sperm count and motility. It is advised that the swim-up method be avoided in cases of ejaculates exhibiting abnormally high ROS production, as might be the case in leukoospermic samples (46). In such cases, lengthy exposure of sperm to the semen may prove to be highly detrimental to sperm integrity. It is therefore preferable that sperm be removed from the seminal plasma as soon as possible following liquefaction, in which case the PureSperm<sup>®</sup> density gradient method or the conventional swim-up from a washed pellet may be acceptable.

### 5.3 Conclusion

In conclusion, results from the first part of this investigation confirm that sperm head dimensions are significantly affected as a result of morphology staining. Results showed that RD-staining caused sperm head swelling while PAP-staining caused sperm-head shrinkage. These contrasting effects are likely to be explained by the differences between the osmolarities of the staining solutions and the semen. No adverse effects on sperm head dimensions were apparent when SB was used, possibly owing to the SB-solutions which were shown to be relatively iso-osmotic to semen. In contrast to RD and PAP, a further favourable characteristic of SB was its ability to effectively stain the acrosome. In general, SB revealed morphometry values closest to which would be expected following the evaluation of fresh unstained sperm. Although results showed few significant differences between the stains with regards to morphology evaluation, there still remains a level of concern surrounding the evaluation of patients with borderline morphology. In such cases, the choice of stain has the potential to cause small alterations to the sperm dimensions which may result in the patient being classified as either normal or abnormal for sperm morphology, depending on the staining technique chosen for evaluation.

On the whole, the SB staining technique was shown in this study to be a simple and rapid procedure, which produces high quality results which allows for easy detection and accurate morphology evaluation by the SCA<sup>®</sup>. Based on the results in this study, SB is a more favourable choice for use in sperm morphology evaluation when compared to RD and PAP staining techniques in terms of quality of results, repeatability and practicality. Since this study only involved the evaluation of sperm head morphology, further investigations ought to assess the effectiveness of the SB staining technique with regards to midpiece and tail evaluation.

In the second half of this study, the efficiency of two commonly used sperm preparation techniques were evaluated in terms of ability to isolate a high quality of sperm. Results showed that while the swim-up method isolated sperm with superior motility and significantly improved head morphology according to WHO criteria, the concentration of the resultant population was very low. The PureSperm<sup>®</sup> density gradient technique on the other hand, showed to improve sperm morphology

according to WHO and Tygerberg strict criteria, while significantly improving several motility parameters and providing a suitable concentration of sperm.

Since each sperm separation technique is associated with its own set of strength and weaknesses, ultimately the method of choice should be based on the requirements of the given fertility treatment (ICSI, IUI, GIFT or IVF) as well as the characteristics of the semen sample to be processed. Based on the results obtained in this investigation it is suggested that with fertility treatments requiring low concentrations of sperm (i.e. ICSI, where only one sperm is required), the swim-up method may be suitable. However, in cases where normal morphology or higher concentrations of sperm are imperative to the success of the fertility treatment (i.e. IUI and IVF), the PureSperm<sup>®</sup> density gradient technique may be a more suitable option. Furthermore, the PureSperm<sup>®</sup> density gradient technique may be more practical option of sperm separation with semen samples associated with high viscosity, low sperm concentration or sperm motility.

It should however be stated that in order to more accurately determine the best semen preparation method for use in a clinical setting, it is essential that the outcome of these techniques (with regards to sperm yield, motility, and morphology) be evaluated against the success rates of the given fertility treatments.

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