Human Reproduction, Vol.25, No.6 pp. 1369–1382, 2010
Advanced Access publication on April 17, 2010 doi:10.1093/humrep/deq075

human reproduction

ORIGINAL ARTICLE *Andrology*

Morphometric dimensions of the human sperm head depend on the staining method used

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Submitted on November 11, 2009; resubmitted on February 14, 2010; accepted on March 3, 2010

BACKGROUND: Assessment of sperm morphology (including morphometry) is extensively used to determine one of the qualities of a semen sample and depends on the differential staining of spermatozoa. A staining technique should cause as little change to sperm dimensions and form as possible in order to reliably evaluate the morphometric features of the sperm. Various staining techniques have been employed, but only a few have been recommended by the World Health Organization and are amenable to automated sperm morphometry analysis. Our study was aimed at comparing the effect of three staining techniques [Papanicolaou (PAP), Rapidiff® (RD) and SpermBlue® (SB)] on human sperm head dimensions and to compare these with the head dimensions in fresh semen.

METHODS: Smears made from human semen samples ($n = 24$) were stained according to the three staining techniques and sperm head morphometry was assessed with the Sperm Class Analyzer. Head dimensions of fresh spermatozoa were measured with a digital calliper on a computer screen. The minimum number of spermatozoa to be analyzed to represent the sperm population and the degree of inter-laboratory variation were determined. Electron micrographs from the same semen samples were used to determine the actual acrosome coverage of the spermatozoa in the semen ($n = 7$) in order to verify the results of the automatic analyses.

RESULTS: The osmolality of human semen differs from that of the RD and PAP fixatives and stains, but is more similar to the SB fixative and stain. At least 100 spermatozoa should be analyzed to include a representative sample of the sperm population. RD caused sperm heads to swell, PAP caused them to shrink and SB had no significant effect on sperm head dimensions when compared with spermatozoa in fresh semen. Very little inter-laboratory variations were found. The percentage acrosome coverage was significantly different between the three staining techniques, as well as between the RD and PAP stains and the manual measurements obtained using the electron micrographs.

CONCLUSIONS: Different staining techniques change the morphometric dimensions of the human sperm head, probably due to the fact that either the fixatives or stains are not iso-osmotic in relation to human semen. Since these changes in sperm head dimensions are not uniform, care should be taken when selecting a staining technique. Ideally, stained spermatozoa should have dimensions as close to spermatozoa in fresh semen as possible, as was found with the SB staining method, resulting in accurate evaluations of sperm head morphometry.

Key words: sperm head morphometry / Papanicolaou / Diff-Quik-like stain / SpermBlue® / automated sperm morphometry analysis

Introduction

The evaluation of male fertility potential is based on the assessment of physical semen characteristics (appearance, liquefaction, volume, pH, viscosity) and standard semen variables (sperm concentration, progressive sperm motility and sperm morphology). Of these semen variables, sperm morphology has been regarded as one of the most important for determining the quality of a semen sample and a male's fertility potential (MacLeod and Gold, 1951; Hartman *et al.*, 1964; Eliasson, 1971; Menkveld and Kruger, 1996; Coetzee *et al.*, 1998).

The value of sperm morphology assessment as a predictor of male fertility has often been challenged due to the existence of different classification systems of normal sperm morphology, various slide

preparation and staining techniques, the inconsistency of the analyses within and between laboratories, and the level of experience of different technicians (Menkveld *et al.*, 1990; Davis and Gravance, 1994; Barratt, 1995; Ombelet *et al.*, 1995; Mortimer and Menkveld, 2001). Different approaches or classification systems for the evaluation of (normal) sperm morphology have and are still being used today, e.g. the liberal approach [MacLeod and Gold, 1951; World Health Organization (WHO) 1980, 1987], the strict (Tygerberg) criteria approach (Menkveld *et al.*, 1990; WHO, 1999), as well as the David *et al.* (1975) and Düsseldorf (Hofmann and Haider, 1985) classification systems. The 1999 and 2010 WHO manuals now recommend that the strict (Tygerberg) approach should be followed for the sperm morphology evaluation process (Menkveld *et al.*, 1990; WHO, 1999, 2010). The recently published WHO reference values of semen characteristics for fertile men (Cooper *et al.*, 2009) should also be used as a guideline.

For a spermatozoon to be considered normal, the whole spermatozoon, i.e. the sperm head, the neck, midpiece and tail, must be normal and no excessive cytoplasmic material may be present (Eliasson, 1971). Therefore, four main categories are noted or distinguished, namely, head defects, neck and midpiece defects, tail defects and presence of cytoplasmic residues (WHO, 1999; Menkveld, 2007). Although the WHO (1999) manual states that 'it is considered unnecessary routinely to distinguish between all the variations in head size and shape or the various midpiece and tail defects', head size and head shape defects are important criteria in the determination of a spermatozoon's morphological normality or abnormality (Eliasson, 1971; Menkveld, 2007). Sperm size and sperm form have been shown to seriously affect normal sperm function, including a spermatozoon's ability to undergo the acrosome reaction (Menkveld *et al.*, 2003) and to bind to the zona pellucida of the oocyte (Garrett *et al.*, 1997). Consequently, these characteristics also have an influence on the prediction of the male's fertility potential (Aziz *et al.*, 1998; Menkveld, 2010).

The accuracy of sperm morphology assessment depends on the careful preparation, fixation and staining of spermatozoa, because these events can, among other things, greatly affect sperm head dimensions and/or sperm head shape, i.e. sperm head morphometry (Davis and Gravance, 1993; Meschede *et al.*, 1993; Menkveld, 2007). Many stains and staining combinations have been employed to assess sperm morphology (Coetzee *et al.*, 1998). Selection of a staining technique not only depends on the fact that it should cause as little change as possible to sperm head morphology and morphometry but also on its ability to stain spermatozoa differentially, clearly indicating the boundaries of the head, acrosome, post-acrosome areas, midpiece and tail, in order to evaluate the normality of each part.

The modified Papanicolaou (PAP) stain has become synonymous with human sperm morphology assessment (Eliasson, 1971; Menkveld *et al.*, 1990; WHO, 1999). However, the modified PAP staining procedure involves in excess of 20 processing steps, uses more than 12 different chemical solutions (WHO, 1999) and is accordingly considered time consuming. The fixation and/or xylene dehydration steps of this staining procedure may also cause shrinkage of the spermatozoon (WHO, 1999). Therefore, some researchers in the field of sperm morphology evaluation have searched for alternative simpler and faster staining methods such as Diff-Quik® (Kruger *et al.*, 1987) or equivalent products such as Hemacolor® (Soler *et al.*, 2003). These staining techniques involve a short fixation period followed by two staining steps in two different staining solutions, each step only taking a few seconds. Despite the findings that Diff-Quik® causes sperm swelling and background staining (WHO, 1999) it is now widely used as a routine procedure for manual assessments as well as for computer aided human sperm morphology assessment (Ombelet *et al.*, 1997; Coetzee *et al.*, 2001).

Recently a new stain, SpermBlue® (SB), has been developed for the evaluation of human and animal sperm morphology (Van der Horst and Maree, 2009). The main advantages of SB are that it involves a simple and fast staining procedure, it stains the various components of spermatozoa differentially, and it is also amenable to automated sperm morphometry analysis (ASMA). SB has, however, not yet been compared with the PAP or Diff-Quik® methods in terms of sperm morphometry measurements. Ideally, these different staining techniques should also be compared with fresh, unstained spermatozoa in order to evaluate the influence of fixation and staining on human spermatozoa.

Several studies have shown the need for ASMA in the routine clinical setting to improve accuracy, reduce subjectivity and reduce intra- and inter-laboratory variation when determining the percentage of normal spermatozoa in a sample (Barroso *et al.*, 1999; Coetzee *et al.*, 1999a, 2001). However, the use of ASMA systems requires standardization of the preparation and staining methods used, as the accuracy of an ASMA system depends on the staining properties of the spermatozoa and the degree of contrast between the spermatozoa and the surrounding background (Coetzee *et al.*, 1999a).

The main aim of the present study was to determine the influence of three different staining procedures on various sperm head morphometry parameters using the automated Sperm Class Analyzer (SCA®) system. To accomplish the former, it was important to first determine the minimum number of spermatozoa that needed to be analyzed to give a representative assessment of sperm head measurements from a specific semen sample, as well as the degree of inter-laboratory variation when using equivalent ASMA equipment. Furthermore, an important aim of this study was to develop an accurate method to measure sperm head length and width in non-fixed and unstained spermatozoa and to compare these measurements with those obtained from stained semen samples.

Materials and Methods

Collection of semen samples

Freshly ejaculated semen samples from 12 donors on various days (total of 24 samples over 3 months) were used in this study. Semen samples were obtained by masturbation after 2–3 days of sexual abstinence. All procedures were in accordance with ethical guidelines of the University of the Western Cape and the research unit where semen was collected (Division of Medical Physiology, Department of Biomedical Sciences, Stellenbosch University). A consent form was signed by each donor participating in the study. Human sperm donor selection was based on sample size (≥2 ml), sperm concentration (≥20 × 10^6/ml) and percentage sperm motility (≥50% forward progression; WHO, 1999) for this particular study. All spermatozoa were measured randomly, to exclude any bias in favour of a specific morphological form.

The osmotic pressure of seven of these semen samples were measured within one to three hours after ejaculation, using a vapour pressure osmometer (Wescor 5100C Vapor Pressure Osmometer, Logan, UT, USA), calibrated with 290 and 1000 mM/kg standard solutions.

Morphometric measurements of spermatozoa in fresh semen

Five randomly selected semen samples were used to measure and determine various sperm head morphometry variables on fresh, unstained spermatozoa. A Basler A312fc digital camera (Microptic S.L., Barcelona, Spain) was mounted (C-mount) on a Zeiss Photomicroscope III (Zeiss, Cape Town, South Africa), equipped with a Neofluar (Ph2) 40 × objective, a 10 × projection lens (ocular) and a 2.0 × Optivar (intermediate lens) providing an 800 × total magnification. Sperm were projected onto a 410 × 260 mm flat screen of the desktop computer using the above mentioned phase-contrast optics, a light setting of 8.5 V and the Manual counter module of the SCA® system version 4.0.0.5 (Microptic S.L., Barcelona, Spain). A Nikon micrometer scale marked in 10 μm units was used for calibration purposes.

A 10 μl drop of thoroughly mixed, liquefied semen was placed on a clean glass slide and covered with a 20 × 20 mm cover slip. By using an electronic digital calliper, measurements of sperm head length and sperm head width were made from the projected spermatozoa on the computer screen as soon as flow underneath the cover slip had stopped (see Fig. 1 for example of method). The electronic calliper was calibrated to 0.01 mm. The average length of projected spermatozoa on the screen was ~13 mm. Three derived semen variables, ellipticity, elongation and the width/length ratio, were calculated from sperm head measurements (see Table I for formulas).

For validation of this method of measurement of the sperm head, three stained slides (one of each staining technique) of the same five randomly selected samples were used (see under 'Staining procedures' below). Manual measurements of the stained spermatozoa with the digital calliper on the screen were compared with the automatic measurements of the identical spermatozoa by the SCA® system. Only sperm head length and sperm head width were measured manually, which allowed the calculation of the ellipticity, elongation and width/length ratio.

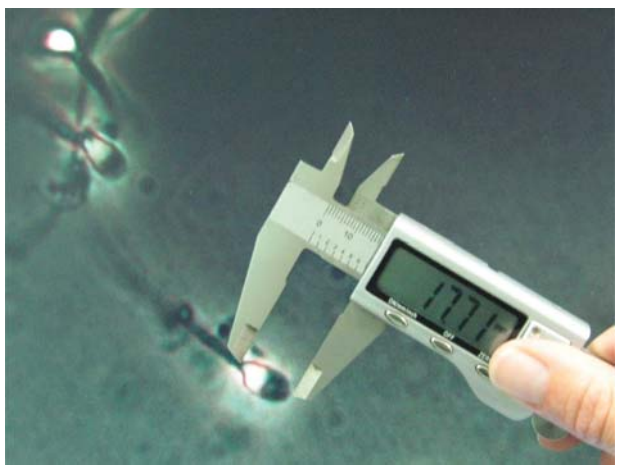

Figure 1 Manual measurement of sperm head variables on the computer screen with a digital calliper.

Table I Morphometric variables of the sperm head measured automatically by the SCA® and manually with a calliper.

Variable	Formula
Length (μm)	L
Width (μm)	W
Perimeter (μm)	P
Area (μm^2)	A
Ellipticity	L/W
Elongation	(L − W)/(L + W)
Roughness	4π (A/P^2)
Regularity	π (L*W/4*A)

Preparation of semen smears

All semen samples were processed for staining within one hour of collection and after liquefaction. The sperm concentration was assessed by pipetting 5 μl of semen into a 20 μm deep chamber of a Leja slide (SC 20-01-04 B; Leja Products B.V., Nieuw-Vennep, The Netherlands), followed by automated analysis with the Motility/Concentration module of the SCA® system. Only 3–5 μl of semen was used to make the smear if the sperm concentration was more than 100×10^6/ml, and ~7–10 μl was used for sperm concentrations between 20 and 90×10^6/ml. This procedure resulted in 2–10 spermatozoa per field viewed using the 100 × oil immersion objective during morphometric analysis. Six slides were made from each semen sample for the different staining procedures and to allow for duplicate slides of each stain.

Chemicals used

The chemicals used during the modified PAP staining procedure (hereafter referred to only as 'PAP staining') were purchased from Merck, Modderfontein, South Africa. The Diff-Quik®-like stain, called Rapidiff® (RD) (identical formulation to Diff-Quik®), was obtained from Clinical Sciences Diagnostics, Southdale, South Africa. SB fixative and stain were supplied by Microptic SL, Barcelona, Spain. All other chemicals were purchased from Sigma-Aldrich, Cape Town, South Africa and only analytical grade or molecular grade chemicals were used.

The osmotic concentration of the stains and their fixatives were measured using the same vapour pressure osmometer as mentioned previously.

Staining procedures

The air-dried smears were fixed in the appropriate fixatives for the recommended time for each of the three staining methods. Immediately after fixation, the slides were stained with the RD stains (Stain 1 and Stain 2; Kruger *et al.*, 1987; WHO, 1999) or the SB stain as previously described (Van der Horst and Maree, 2009). The smears prepared for the PAP staining (WHO, 1999) were stained by clinical technologists in a routine clinical andrology laboratory (Andrology Laboratory, Department of Obstetrics and Gynaecology, Tygerberg Academic Hospital and Stellenbosch University, Tygerberg, South Africa).

Automated sperm morphometry analysis

A Basler A312fc digital camera (Microptic S.L., Barcelona, Spain) was mounted (C-mount) on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa), equipped with bright field optics. The stained spermatozoa were analyzed using a blue filter, a 100 × oil immersion objective and the Morphology module of the SCA® system. The brightness and

contrast settings on the SCA® system were identical for all the ASMA analyses, but the light setting of the microscope was optimized for each staining method. All spermatozoa which did not overlap with each other or with background staining were considered for analysis.

The SCA® system automatically detects the acrosome, head and midpiece of spermatozoa and makes rapid and accurate measurements of various variables. For the purpose of this study, only the acrosome and head variables were included in the evaluations. A total of nine morphometric variables (sperm head length, head width, head perimeter, head surface area, head ellipticity, head elongation, head roughness, head regularity and the percentage acrosome coverage of the head) were either assessed or calculated. A 10th derived variable (the width/length ratio, as recommended by Davis and Gravance, 1994), was also calculated and used in the statistical analysis. Table I summarizes the various morphometric measurements and the formulas used by the SCA® system for the derived variables.

Number of spermatozoa to be analyzed

Six stained slides (two RD, two PAP and two SB slides) were randomly selected from the pool of 12 donors and spermatozoa were analyzed in four sets of 50 spermatozoa per slide. Each set of 50 randomly selected spermatozoa were followed by a different set of 50 randomly selected spermatozoa, until a total of 200 spermatozoa had been analyzed per slide. During the statistical analysis, the four sets of 50 spermatozoa were compared with each other to determine if there were differences in selecting any random number of 50 spermatozoa. The first two and the last two sets of 50 spermatozoa were also combined (creating two sets of 100 spermatozoa each) to determine if there were differences in selecting any random number of 100 spermatozoa. Only sperm head length, width, perimeter and acrosome coverage were used to determine the number of sperm to be analysed since these variables were subsequently used to derive the other sperm head variables (Table I).

Taking the results of the above into consideration, at least 100 spermatozoa were analyzed for each of the three different stained smears per semen sample and at least 50 spermatozoa for the wet fresh measurements of spermatozoa.

Inter-laboratory variation using ASMA

The inter-laboratory variation was determined by using two equivalent SCA® systems (operated by two independent technicians) to analyze the same stained slides. The above mentioned ASMA setup was used at the Department of Medical Biosciences, University of the Western Cape, South Africa. A similar setup was used by the Division of Medical Physiology, Department of Biomedical Sciences, Stellenbosch University, South Africa, with the only difference being that a different model microscope, a Nikon Eclipse E200 (IMP, Cape Town, South Africa), was used instead of the Nikon Eclipse E50i.

Transmission electron microscopy

Semen samples were prepared for transmission electron microscopy (TEM) using standard preparation techniques (Chemes *et al.*, 1998). This involved fixing 200–400 µl semen in 500 µl 2.5% phosphate buffered glutaraldehyde followed by post-fixation in 1% osmium tetroxide in the same buffer. The fixed samples were subsequently processed for TEM including contrasting with lead citrate and uranyl acetate. A Reichert ultramicrotome (SMM Instruments, Johannesburg, South Africa) with a diamond knife (Agar Scientific, Randburg, South Africa) was used to make silver to gold sections. Thin sections on copper grids were examined using a Jeol JEM 1011 transmission electron microscope at 80 kV (Advanced Laboratory Solutions, Johannesburg, South Africa). The electron micrographs of seven donor samples were assessed to determine the acrosome coverage of the human sperm head. In this regard, only spermatozoa sectioned in sagittal or coronal planes were analyzed. After taking actual measurements of the length of the sperm head and acrosome, the relevant percentage acrosome cover (acrosome length/sperm head length) could be determined. This data was used to compare it to the percentage acrosome coverage determined by the SCA® system for the three different staining techniques.

Statistical analysis

The MedCalc programme version 9.3.6.0 (Mariakerke, Belgium) was used for all statistical analyses. Tests were performed for normality of distribution. Data sets mostly represented normal distributions. Accordingly, one way analysis of variance (ANOVA) was used to compare more than two sets of data. In this respect Levene's test for equality of variances was applied and when $P > 0.05$, ANOVA analysis was performed. Any significant differences ($P < 0.05$) as indicated in the ANOVA table between groups were furthermore analyzed using the Student–Newman–Keuls test for all pairwise comparisons. In subsets of data that appeared to have non-parametric data distributions, the Kruskal–Wallis test was employed and further elaborated for individual differences using the Mann–Whitney *U*-test for independent samples. Bland and Altman plots were used to establish the validity of the manual method of measurements of spermatozoa in fresh semen compared with both the automatic and manual measurement of stained smears (all three stains) of the same donor. Passing and Bablok linear regression graphs were used to verify that the manual measurements obtained with the calliper on screen and the automatic measurements were due to accurate/inaccurate measurement and not due to chance. Data are represented as the mean ± standard deviation (SD) in the tables and $P < 0.05$ was considered significant. In the figures where data are represented as box-and-whisker plots, each box plot displays the following parameters for a given distribution: (i) median (centre line of box), (ii) second and third quartile values representing the middle 50% of the values (central box), (iii) range of data excluding data points lying outside the one and a half times interquartile range (T-bars) and (iv) data points lying outside the one and a half time (plotted with a square marker) or three times the interquartile range (plotted with a round marker).

Results

Osmolality

The semen characteristics and the osmolality of human semen, fixatives and stains used during this study are shown in Table II. It is evident from these values that there are distinct differences among

Table II Semen characteristics (average ± SD) and osmolality of semen, fixatives and stains.

Semen variable	Measurement
Volume (ml)	3.3 ± 0.9
Sperm concentration (10^6/ml)	56.8 ± 19.8
Motility (%)	66.5 ± 18.4
Osmolality (mOsm/kg or mM/kg):	
Semen ($n = 7$)	360
Rapidiff® Fixative	46
Rapidiff® Stain 1	182
Rapidiff® Stain 2	170
Methanol	82
SpermBlue® Fixative	319
SpermBlue® Stain	377

the semen osmolality (360 mM/kg) and that of the PAP methanol fixative (82 mM/kg), the RD fixative (46 mM/kg) and the two RD stains (182 and 170 mM/kg). The SB fixative (319 mM/kg) and SB stain (377 mM/kg) had osmolality values much closer to that of the semen.

Influence of the number of spermatozoa analyzed

Comparison of the variation within the four sets of 50 spermatozoa or the two sets of 100 spermatozoa revealed no significant difference (Levene's test, $P > 0.05$) for all three staining techniques (data not shown), when the sperm head length, width and perimeter were assessed. This was also true when the different groups (sets of 50 or 100 spermatozoa) were compared with each other (ANOVA, $P > 0.05$) (Fig. 2, data for the two sets of 100 spermatozoa are not shown). However, when the acrosome coverage was assessed, there was significant variation between the four sets of 50 spermatozoa for RD and SB (the first set of 50 spermatozoa was significantly different from the other sets). Once again, there was no significant variation within the groups or any significant difference among the

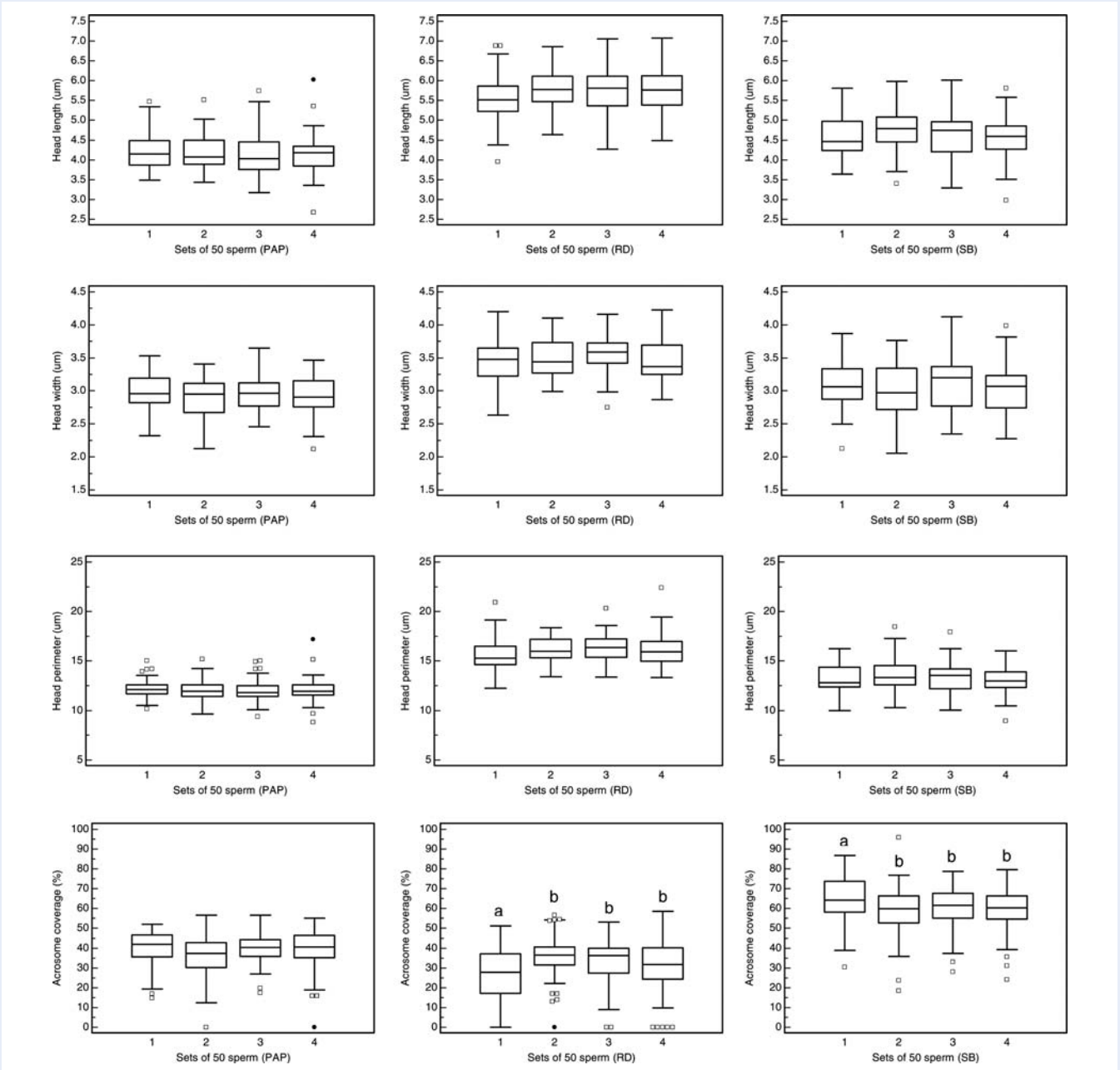

Figure 2 Box-and-whisker plots illustrating the variation found within and between different sets of 50 sperm for selected sperm head variables. 1, 2, 3, 4 = different sets of 50 sperm (1 = 1–50, 2 = 51–100, 3 = 101–150, 4 = 151–200). PAP, Papanicolaou; RD, Rapidiff®; SB, SpermBlue®. a, b = boxes labelled with different letters are significantly different ($P < 0.05$) from each other within the staining technique.

groups when acrosome coverage was assessed for PAP and for the two sets of 100 sperm of RD or SB. It is assumed that if no significant difference was found between the four sets of 50 spermatozoa or the two sets of 100 spermatozoa, there will also be no difference between any set of 50, 100, 150 or 200 spermatozoa. Thus, in general, it should be sufficient to analyze only 50 spermatozoa to have a representative sample of the population, but it is recommended that 100 spermatozoa be analyzed, especially if the acrosome coverage is of importance.

Influence of staining procedure on sperm head morphometry

The morphometric values of the various sperm head variables according to the three staining procedures are presented in Table III. All these variables were significantly influenced by the staining procedure ($P < 0.05$), however, the differences between the three staining procedures revealed various trends. Although Table III only includes the mean values, the same significant differences and trends were found among the three staining procedures for each donor.

For the sperm head length, width, area and perimeter measurements (Fig. 3), RD had significantly larger values, followed by SB and then PAP i.e. RD > SB > PAP, except for width, where there was no significant difference between SB and PAP. In terms of acrosome coverage, SB measurements showed a significantly larger percentage coverage, followed by PAP and then RD (SB > PAP > RD). SB also had the highest value for elongation and ellipticity, but there was no significant difference between the other two staining procedures for these variables. PAP had the lowest value for regularity, but the highest value for roughness ($P < 0.05$).

Figure 4 shows examples of human semen stained according to the three staining procedures and analyzed by means of the SCA® Morphology module. For each spermatozoon analyzed, the actual stained spermatozoon is shown on the left, and on the right the analysis of the same spermatozoon (acrosome yellow, head blue and midpiece green) is superimposed on it. It is evident from this visual evaluation that there is a higher degree of background staining in RD, with many other stained particles in close contact with the stained sperm head. The differences in the measurements of the various sperm head variables among the three staining procedures, as shown in Table III and Fig. 3, are also seen in Fig. 4, especially the differences in the head length, width and percentage acrosome coverage.

Inter-laboratory variation when using equivalent ASMA systems

In general, there were no major significant differences in the automatic analyses of the various sperm head variables between the two laboratories involved (Fig. 3). For three variables (head length, head perimeter and acrosome coverage), however, differences were found when the RD stain was evaluated. The acrosome coverage measured in the SB stained spermatozoa was also different between the two laboratories.

Morphometric measurements of spermatozoa in fresh semen

A comparison of the manual measurements (calliper on the screen) with the automatic measurements (SCA® system) for sperm head length and width were used to validate the method utilized for measuring spermatozoa in fresh semen (Fig. 1). The Bland and Altman plots in Fig. 5 indicate that the data points are evenly distributed and that there were no distinct deviations from the mean for any of the three staining procedures. Calculation of the Passing and Bablok regressions (Fig. 5) reveals no significant deviation from linearity ($P > 0.10$). Although Fig. 5 only indicates the sperm head length variable, the same distributions and regressions were found for the sperm head width. Therefore, since there were no significant differences between the manual and automatic analyses of the sperm head length and width, it was validated that this method (calliper on screen) of measuring spermatozoa in fresh semen is an acceptable method for the measurement of these variables.

Table III also includes the measurements of selected sperm head variables in fresh semen compared with the values obtained by the

Table III SCAR analysis (mean ± SD) of sperm head morphometry for the three staining techniques compared with spermatozoa in fresh semen.

	Papanicolaou	Rapidiff®	SpermBlue®	Fresh measurement*
Number of semen samples	24	24	24	5
Length (μm)	4.28 ± 0.27^{a}	5.17 ± 0.27^{b}	4.73 ± 0.27^{c}	4.79 ± 0.26
Width (μm)	2.65 ± 0.19^{a}	3.12 ± 0.21^{b}	2.75 ± 0.24^{a}	2.82 ± 0.23
Area (μm^2)	9.26 ± 0.99^{a}	12.87 ± 1.19^{b}	10.47 ± 1.21^{c}	–
Perimeter (μm)	11.83 ± 0.69^{a}	14.33 ± 0.75^{b}	12.99 ± 0.80^{c}	–
Ellipticity	1.63 ± 0.11^{a}	1.68 ± 0.10^{a}	1.75 ± 0.13^{b}	1.73 ± 0.12
Width/length ratio	0.62 ± 0.04^{a}	0.60 ± 0.04^{a}	0.58 ± 0.04^{b}	0.59 ± 0.04
Elongation	0.23 ± 0.03^{a}	0.25 ± 0.03^{a}	0.27 ± 0.03^{b}	0.26 ± 0.03
Roughness	0.83 ± 0.02^{a}	0.79 ± 0.02^{b}	0.78 ± 0.03^{b}	–
Regularity	0.96 ± 0.01^{a}	0.98 ± 0.01^{b}	0.97 ± 0.02^{b}	–
Acrosome coverage (%)	32.76 ± 7.43^{a}	23.73 ± 7.97^{b}	46.29 ± 8.63^{c}	–

[a,b,c]Values with different superscript letters in the same row are significantly different ($P < 0.05$) from each other.
*Significance values are shown in Fig. 6.

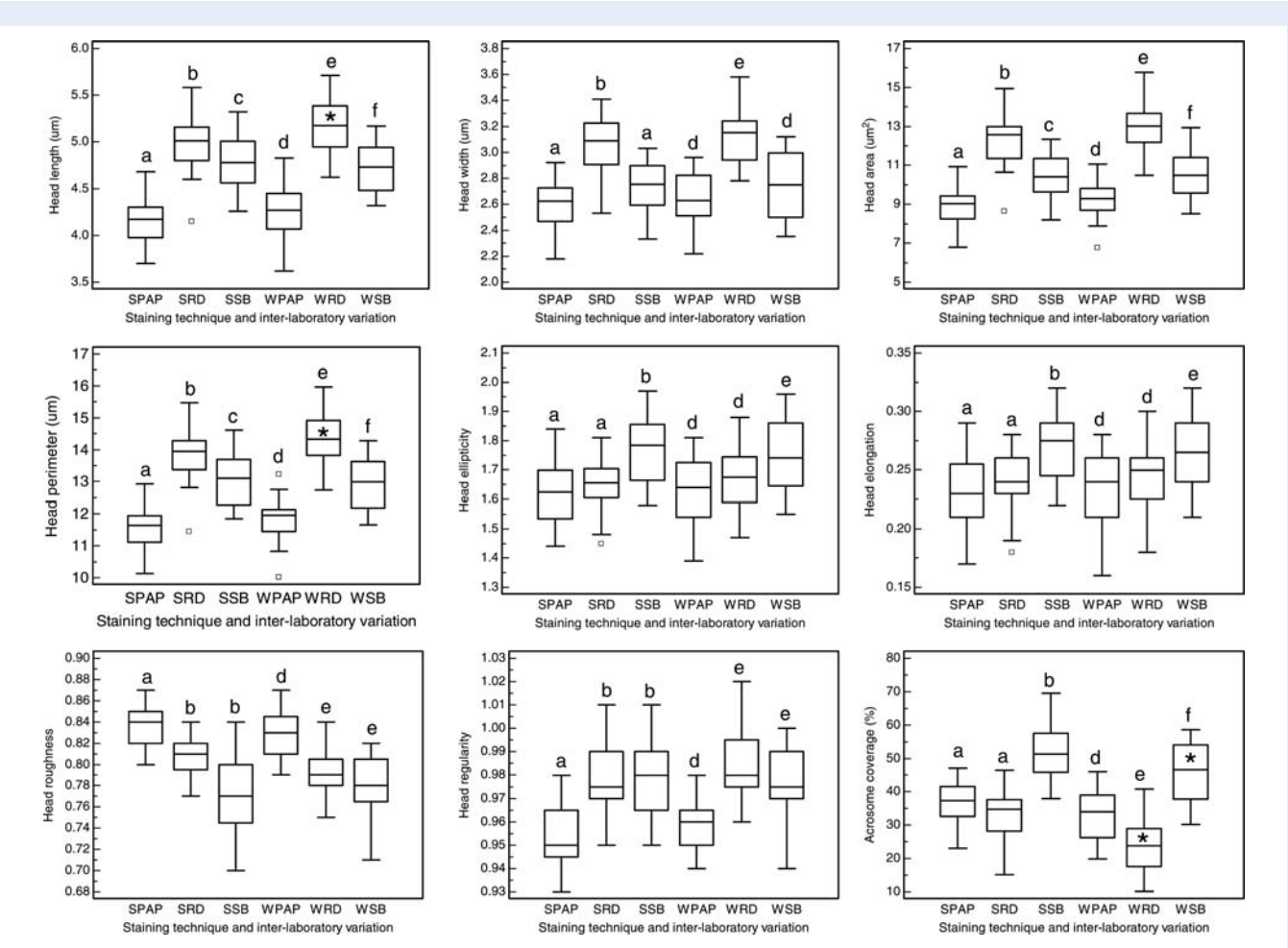

Figure 3 Box-and-whisker plots illustrating the influence of staining technique on various sperm head variables (donors $n = 24$).

The three staining techniques include PAP, RD and SB. The inter-laboratory variation is also indicated—'S' and 'W' in front of the staining technique refers to the two institutions where these analyses were performed using equivalent ASMA systems. a, b, c = boxes labelled with different letters are significantly different ($P < 0.05$)—institution 'S'. d, e, f = boxes labelled with different letters are significantly different ($P < 0.05$)—institution 'W'. * = significant inter-laboratory variation ($P < 0.05$).

SCA® system for the three staining procedures, using five randomly selected donors (all part of the original 12 donors). Figure 6 includes comparisons of selected sperm head variables of the fresh and stained spermatozoa of the same five donors. In terms of sperm head length, the PAP stained spermatozoa were significantly shorter than the fresh spermatozoa, as well as the RD and SB stained spermatozoa (Fig. 6). On the other hand, the RD stained spermatozoa were significantly wider than the PAP-stained, SB-stained and fresh sperm (Fig. 6). In contrast, fresh measurements did not differ significantly from SB measurements for sperm width or length ($P > 0.05$; Fig. 6). Furthermore, there was no difference between the ellipticity, elongation or width/length ratio of the fresh measurements and any of the stained sperm measurements ($P > 0.05$; Fig. 6).

TEM acrosome coverage of the human sperm head

Figure 7 contains examples of sagittal sections through the human sperm head, revealing the posterior end of the acrosome. These electron micrographs clearly show that the acrosomal region covers more than 40% of the length of the sperm head. Figure 8 indicates the differences found between the acrosome coverage of the human sperm head as analyzed by the SCA® system for the three staining techniques, compared with the manual measurements of the corresponding donors' acrosome coverage using the electron micrographs. PAP stained spermatozoa had a mean percentage acrosome coverage of 33.8 (±6.73), RD 24.2 (±8.55), SB 47.4 (±6.86) and the manual measurement was 54.6 (±3.22; Fig. 8).

Discussion

In the present study on the morphometric dimensions of the human sperm head, it was found that different staining techniques did have an influence on these dimensions. The changes in sperm head dimensions were not uniform when the effect of three staining techniques was compared with spermatozoa in fresh semen. In terms of the differential staining of the various parts of the spermatozoon, it was also shown that the three staining techniques did not reveal the same boundaries of the acrosome, as measured by the SCA® ASMA system.

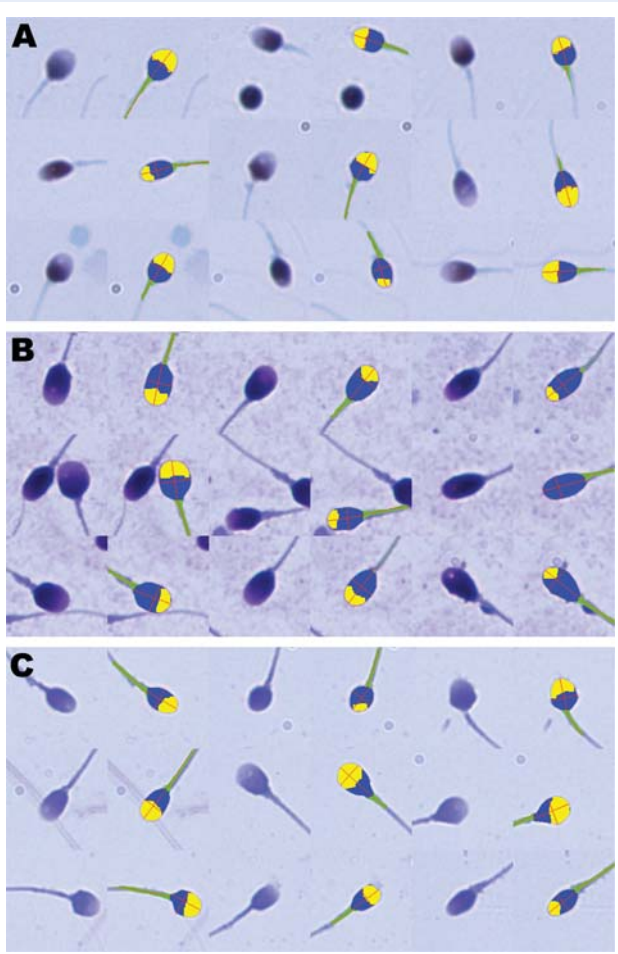

Figure 4 SCA® analysis of the same human semen sample stained with PAP (**A**), RD (**B**) and SB (**C**).

The SCA® 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® analysis of that particular sperm is shown. Actual measurements of the effect of the staining techniques on the sperm head dimensions are shown in Table III.

The search for a staining technique which is both fast and easy and stains spermatozoa differentially for morphology analysis has been an ongoing process (Coetzee *et al.*, 1997; Ombelet *et al.*, 1997). With the increasing use of automated systems for sperm morphology and morphometry analysis, many of these widely used staining techniques have been re-evaluated for their application in ASMA (Davis and Gravance, 1993; Coetzee *et al.*, 1997; Menkveld *et al.*, 1997; Soler *et al.*, 2003). Once a new staining method has been developed, it should also be compared with these well recognized staining procedures to determine its suitability for sperm morphology and morphometry analysis. Another important factor that should be taken into account when selecting a staining technique is how the staining procedure alters sperm morphology and/or the sperm dimensions compared with those found in fresh semen.

Two factors that have an influence on sperm dimensions are the osmolality and the tonicity of the surrounding medium. In studies which focused on the cryopreservation of spermatozoa, it has been reported that spermatozoa act as osmometers and will swell when placed in hypotonic media and shrink in hyper-osmotic conditions (Drevius, 1972; Gao *et al.*, 1993). Sperm motility and membrane integrity are also affected by these two factors and seem to be more sensitive to hypertonic than hypotonic conditions (Rutlland *et al.*, 2003). Although it is recommended that culture media for sperm preparations have an osmolality of 280–290 mM/kg (Mortimer, 1994), the osmolality of human semen was determined as ranging between 330 and 370 mM/kg. Compared with the different fixatives and stains used during this study, it is clear that the RD chemicals and methanol (fixative for PAP staining procedure) could cause extreme hypo-osmotic conditions. The influence of each of the more than 12 different chemicals used during the PAP staining technique was not determined. However, it has been reported that xylene, alcohol and the dehydration of human spermatozoa cause shrinkage of cells (Ross, 1953; Gao *et al.*, 1993) and that different fixative techniques alter sperm head dimensions significantly (Sancho *et al.*, 1998). According to the osmolalities reported in this study and the composition of the SB fixative and stain, these media are relatively iso-osmotic and isotonic (due to balanced salts solutions) in relation to the semen and, therefore, should have less effect on the sperm head dimensions than RD and PAP.

The number of spermatozoa to be analyzed to include a representative sample of the whole population varies between the different techniques employed to analyze spermatozoa. If sperm morphology is determined manually, it is recommended to evaluate at least 200 spermatozoa per sample (WHO, 1999). The implementation of ASMA systems has increased the accuracy and objectivity of sperm morphology analysis (Coetzee *et al.*, 1999a) and, therefore, it is suggested that fewer spermatozoa (50 or 100) need to be analyzed per sample to represent the sperm population. However, since the acrosome coverage was significantly different between the sets of 50 spermatozoa for RD and SB in the present study, it would be advised to analyze at least 100 spermatozoa when using these two staining procedures. This number of spermatozoa to be analyzed is consistent with other reports which also implemented the SCA® system or similar staining techniques (Gago *et al.*, 1998; Coetzee *et al.*, 1999a; Soler *et al.*, 2003).

A high incidence of background staining was found with the RD staining technique, which caused the boundaries of the sperm head to be masked and not easily detected by the SCA® system. This resulted in incorrect analyses and an increase in the time of analysis for these stained slides, because a large number of spermatozoa had to be eliminated. Similar problems with background staining have been reported when either the PAP or Diff-Quik® staining techniques have been used (Kruger *et al.*, 1995; Menkveld *et al.*, 1997; Gago *et al.*, 1998). Very little interference or background staining were experienced with the PAP or SB stained slides in the present study. One option to reduce the background staining would be to wash the semen before preparation of the smears (Kruger *et al.*, 1996; Lacquet *et al.*, 1996). Since most routine semen analysis is performed using unprocessed semen (WHO, 1999), all analyses in the present study were done on unwashed spermatozoa from the neat ejaculate.

The effect of different staining techniques on sperm morphology and morphometry has been investigated (Davis and Gravance, 1993; Lacquet *et al.*, 1996; Oral *et al.*, 2002). A few studies acknowledge the fact that some staining techniques either increased or decreased the sperm head dimensions (Katz *et al.*, 1986; Meschede *et al.*, 1993; Franken *et al.*, 2000; Hidalgo *et al.*, 2006), but that in the case of

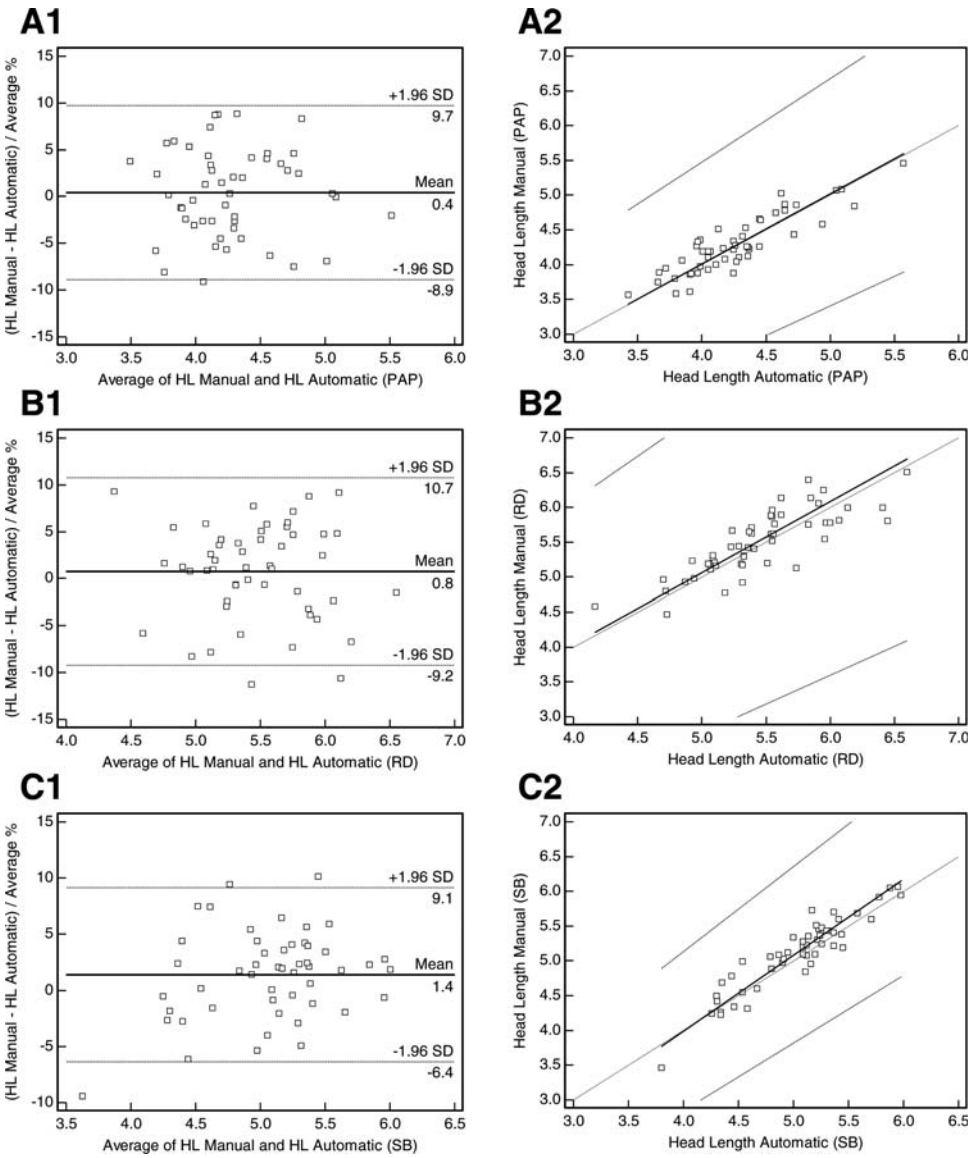

Figure 5 Bland and Altman plots (**A1**, **B1**, **C1**) with corresponding Passing and Bablok regression graphs (**A2**, **B2**, **C2**) for the comparison of the manual and automatic analysis of sperm head length (HL).

PAP, Papanicolaou; RD, Rapidiff®; SB, SpermBlue®

ASMA analyses, these systems should be adapted to incorporate the changes in dimensions (Menkveld *et al*., 1996; Coetzee *et al*., 1997; Henkel *et al*., 2008). In the present study, RD stained cells had the highest values for head length, width, area and perimeter, although the opposite was true for PAP stained spermatozoa. The values for SB stained spermatozoa fell in between that of the other two staining techniques (RD > SB > PAP). The fact that the SB stained spermatozoa had significantly different ellipticity and elongation, was due to the fact that the difference in the sperm head length and width, expressed as a percentage of length or width, was the highest for SB (42%, 72%) (RD 40%, 66%; PAP 38%, 61%). The measurements of the various human sperm head morphometry parameters determined in the present study fall within the range reported by Katz *et al*. (1986) and Soler *et al*. (2003) for PAP and Diff-Quik® staining.

It was clearly shown that in terms of head length and width the SB stained sperm values were most consistently similar to the fresh measurements of native spermatozoa. RD caused excessive swelling in terms of the width of the sperm head and PAP caused the sperm head to shrink in terms of length when compared with the fresh measurements. Although the RD stained spermatozoa did not differ from the SB stained spermatozoa and the fresh spermatozoa in terms of sperm head length, it still had the largest value for this parameter. Similarly the PAP stained spermatozoa still had the lowest value in terms of sperm head width (RD > SB > PAP). The difference in the head length and width, expressed as a percentage of length or width, for SB (42%, 72%) was closest to the values for the native spermatozoa (41%, 70%). Thus, looking at the overall changes in sperm head dimensions, it seems that RD causes spermatozoa to swell and PAP causes

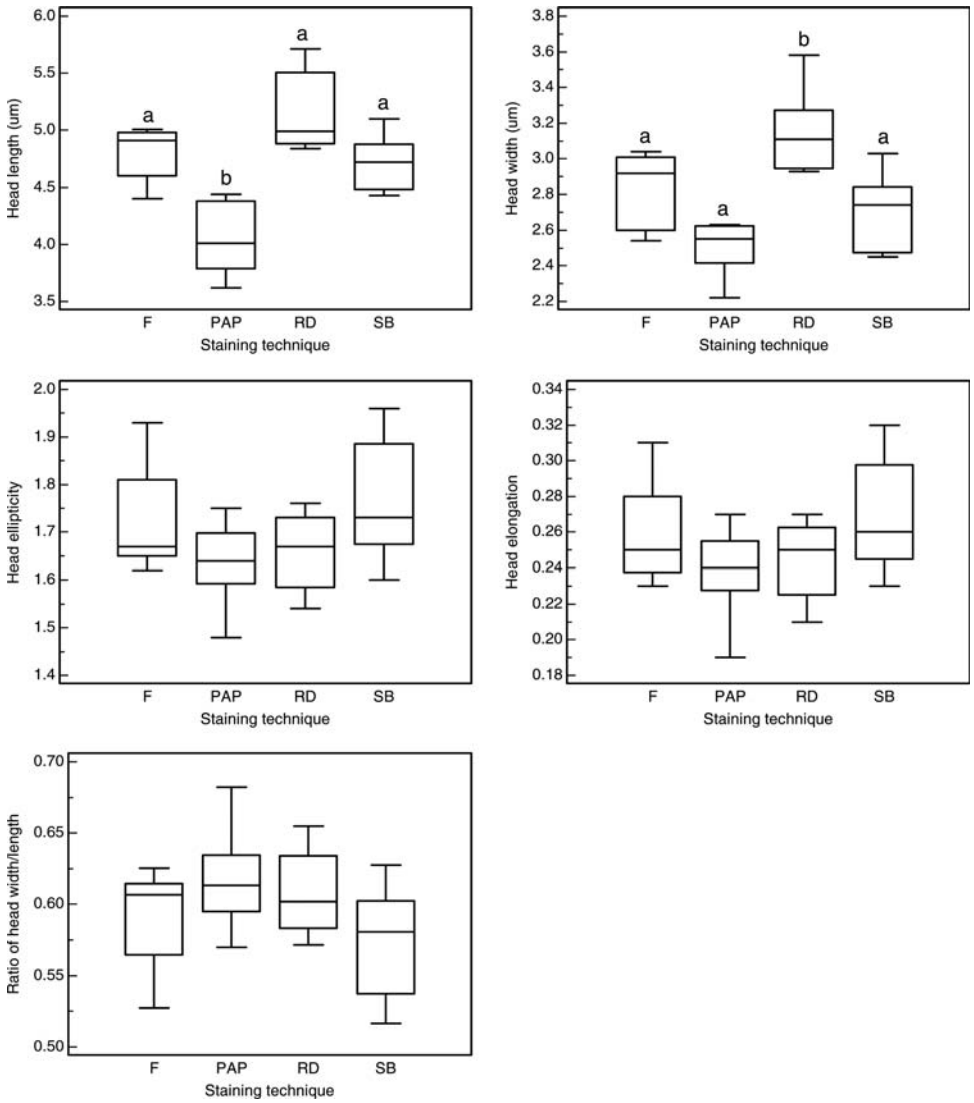

Figure 6 Box-and-whisker plots illustrating the comparison of PAP, RD and SB staining techniques with the fresh measurement (F) of selected sperm head variables (donors $n = 5$).

The actual measurements of these variables are shown in Table III. a, b, c = boxes labelled with different letters are significantly different ($P < 0.05$).

spermatozoa to shrink. Similar effects have been reported in previous studies that investigated Diff-Quik®-like stains (Gago *et al.*, 1998; Soler *et al.*, 2003) and PAP (Katz *et al.*, 1986; Davis and Gravance, 1993). No adverse effects on sperm head dimensions were reported when SB was used to stain spermatozoa from various species (Van der Horst and Maree, 2009) and this has been indicated again in the present study.

When comparing the osmolality values of the different fixatives and stains with the effects of the three staining techniques on sperm head dimensions, it is evident that these factors influence each other. The question, however, still remains that, if the changes in sperm head dimensions caused by the different staining techniques are not uniform, will all spermatozoa be affected to the same degree and may this cause a higher or lower percentage scoring of normal or abnormal spermatozoa in a semen sample? Hidalgo *et al.* (2006) suggested that the extent of the changes due to a specific staining technique might cause the spermatozoa to become more uniform in size and shape, resulting in lower variability within the sperm head measurements. If these changes in the sperm head dimensions cause the spermatozoa to become more or less 'normal' according to the different classification systems, it could result in an increase or decrease in the percentage normal spermatozoa. For instance, several studies reported that the Diff-Quik® method resulted in a higher percentage normal sperm morphology when directly compared with other staining methods (Menkveld *et al.*, 1997; Coetzee *et al.*, 2001). Since the percentage normal spermatozoa in a semen sample is widely used as an indication of a patient's fertility status (Enginsu *et al.*, 1991; Hofmann *et al.*, 1996; Coetzee *et al.*, 1999b), this could theoretically result in an incorrectly diagnosed infertility or subfertility status and possible infertility treatment.

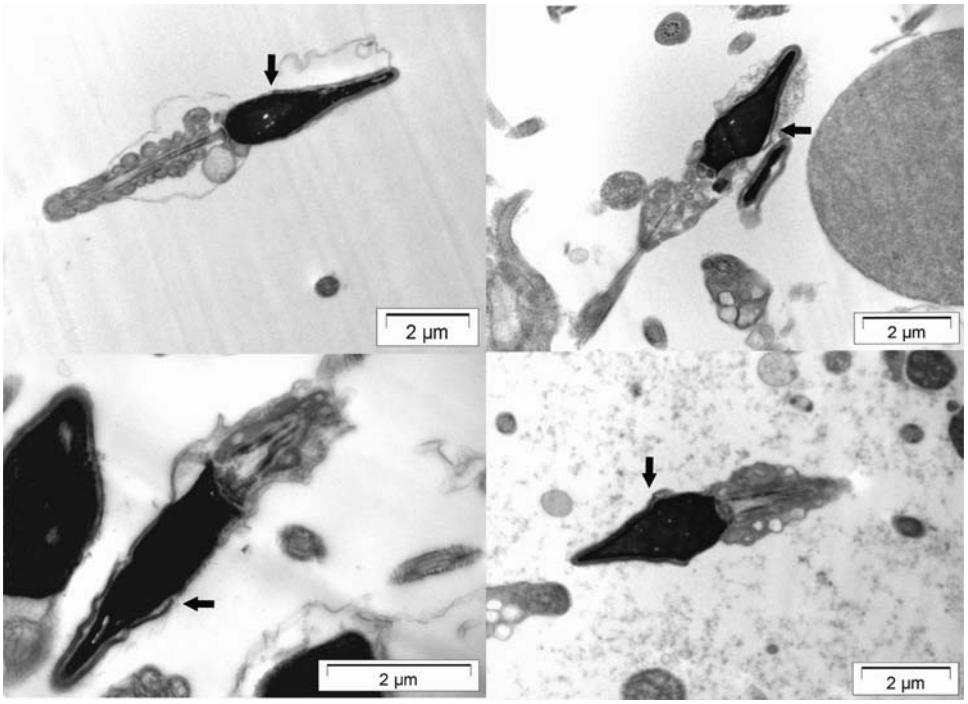

Figure 7 Electron micrographs of sagittal sections through the human sperm head—arrows indicate the posterior end of the acrosome.

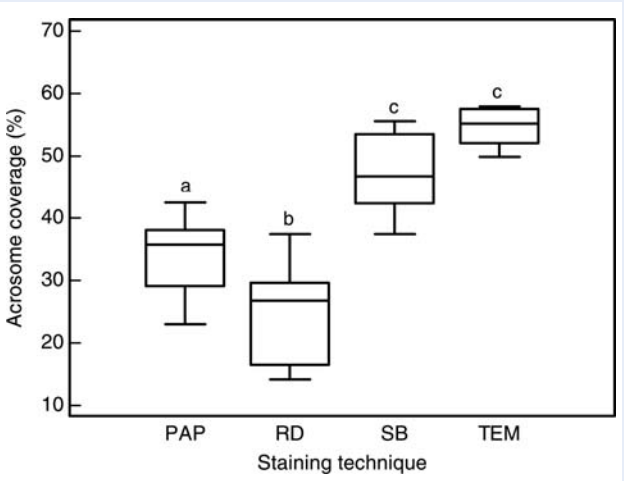

Figure 8 Box-and-whisker plot to illustrate the comparison of the percentage acrosome coverage of the sperm head as analyzed by the SCA® system for the three staining techniques (PAP, RD, SB) and the manual measurement of electron micrographs (TEM) (donors $n = 7$). a, b, c = boxes with different letters are significantly different ($P < 0.05$).

Another important parameter which seems to be influenced by the staining technique is the acrosome coverage of the sperm head. The significant differences found between the acrosome coverage of the three staining techniques substantiates the fact that different stains and staining procedures do not result in identical acrosomal boundaries. The most notable fact is, however, that when the same semen samples were evaluated (measured with the SCA® system), the SB stained spermatozoa had a mean acrosome coverage of 47.4%, PAP 33.8% and RD 24.2%. In the WHO guidelines for semen analysis (WHO, 1999) it is stated that acrosome coverage of 40–70% should be considered as normal. This essentially means that if the spermatozoa were stained with SB, it would be classified normal, but abnormal if stained with PAP or RD.

Verification of the actual size of the acrosome with electron microscopy revealed that the mean acrosome size is 54.6% of the sperm head, which indicates that the SB values for the acrosome coverage is closest. One criticism of using this manual method to verify the percentage acrosome cover could be that only the length of the acrosome and sperm head was measured to determine the percentage cover, whereas the SCA® system determines this parameter by using the area of the acrosome and sperm head. These two methods for determining the percentage acrosome cover should result in nearly identical values if the sperm head is oval or round, but might become problematic if spermatozoa have abnormal shaped heads, e.g. pyriform or elongated (tapering), where the area of the acrosome is larger or smaller relative to the area of the sperm head, but it has no effect of the length of the acrosome. However, when manually measuring the length of the acrosome and sperm head of the spermatozoa analysed by the SCA® system (to validate using the head length rather than the head area), the percentage acrosome coverage was very similar to the values determined by the SCA® system for all three staining techniques (34.6 versus 33.8% for PAP, 26.6 versus 24.2% for RD and 46.0 versus 47.4% for SB). It must be noted that in previous studies a much higher acrosome coverage have been reported with both the Diff-Quik® and the PAP staining techniques (Soler *et al.*, 2003). This could be due to inter-laboratory differences in the execution of the staining

protocol, modifications made to the staining techniques (Ombelet *et al*., 1997), or due to different equipment used for the analyses.

The credibility of the methods used and the results obtained with ASMA during the present study were proven with the low inter-laboratory variation found between the two institutions involved. Since the same slides were analysed by the two institutions, the significant difference found in the sperm head length for the RD stained spermatozoa could be due to the fact that by chance the sperm populations selected during analyses were not similar to each other, or that RD staining led to differences in sperm dimensions and is therefore less reliable or repeatable. The significant difference found between the acrosome coverage of RD and SB stained spermatozoa between the two institutions could be due to different light intensities selected, causing the acrosome to be illuminated differently. Nevertheless, irrespective of this inter-laboratory difference, there were still significant differences between the acrosome coverage of the three staining techniques when evaluated by the two institutions independently.

In conclusion, the morphometric dimensions of the human sperm head were influenced by the different staining procedures, probably due to the difference in the osmolality of the semen and that of the respective fixatives and stains. In terms of differential staining, the acrosome was best differentiated by SB, although RD and PAP had much lower values for the acrosome coverage. On the basis of our findings, the head morphometry of SB stained spermatozoa is closest to that of the native sperm. In contrast, the sperm head measurements of PAP stained spermatozoa were consistently smaller than, and the head measurements of the Diff-Quik-like stained spermatozoa consistently larger than the measurements of SB stained and native sperm. It also appears that neither shrinkage nor swelling of spermatozoa occurs evenly in the length or the width direction, e.g. PAP staining shrinks the sperm head in the length but not in the width. Although this study was not concerned with normal sperm morphology *per se*, when scoring spermatozoa manually, a spermatozoon may be classified 'abnormal' or 'normal' on the basis that the head was shrunken or swollen out of proportion. The authors accordingly recommend that the various manufacturers of ASMA equipment pay special attention to the sperm head size differences for different staining techniques, as quantified in this study, and adapt their values to be able to correctly evaluate spermatozoa in future.

This is the first study that made a serious effort to measure the actual head length and width of native sperm in semen. This method of measurement is not only new and accurate, but it also provides an extremely important baseline study both for future reference and to compare the effect of different staining techniques to fresh measurements of spermatozoa in semen. Future studies could also focus on comparing the current three staining techniques in terms of assessment of the percentage normal spermatozoa, their application with different classification systems, e.g. WHO (1987) and Tygerberg Strict Criteria (Menkveld *et al*., 1990; WHO, 1999) and their relationship with other sperm characteristics.

Authors' roles

G.vdH. led the study and all authors contributed to the study design, data analyses and collation of the results. G.vdH. provided statistical knowledge and L.M. drafted the paper. All authors contributed to the interpretation of the data and were involved in the critical review of the paper.

Acknowledgements

The authors express their gratitude to Ms D. McAlister and Dr F. Lampiao, Division of Medical Physiology, Department of Biomedical Sciences, Stellenbosch University, South Africa, for their technical assistance during the preparation of the semen samples and the analyses of the stained slides. Mr F. Stander and colleagues, Andrology Laboratory, Department of Obstetrics and Gynaecology, Tygerberg Academic Hospital and Stellenbosch University, South Africa, are thanked for the staining of the slides with the Papanicolaou technique. Ms N. Muller, Electron Microscopy Unit, Tygerberg Hospital, South Africa, is thanked for her assistance with the preparation of the TEM samples and the electron micrographs.

Conflict of interest: G.vdH. collaborates with Microptic SL, Spain, the company who supplied one of the stains included in the study (SpermBlue®) and from whom the SCA® systems were purchased. In our research, the SCA® system was merely used as a tool to test the applicability of automated sperm morphometry assessment (ASMA) in terms of three different staining techniques.

Funding

This study was supported by the Senate Research Fund of the University of the Western Cape and the Harry Crossley Foundation (Stellenbosch University).

References

Aziz N, Fear S, Taylor C, Kingsland CR, Lewis-Jones DI. Human sperm head morphometric distribution and its influence on human fertility. *Fertil Steril* 1998;**70**:883–891.

Barratt CLR. On the accuracy and clinical value of semen laboratory tests. *Hum Reprod* 1995;**10**:250–252.

Barroso G, Mercan R, Ozgur K, Morshedi M, Kolm P, Coetzee K, Kruger T, Oehninger S. Intra- and inter-laboratory variability in the assessment of sperm morphology by strict criteria: impact of semen preparation, staining techniques and manual versus computerized analysis. *Hum Reprod* 1999;**14**:2036–2040.

Chemes HE, Olmedo SB, Carrere C, Oses R, Carizza C, Leisner M, Blaquier J. Ultrastructural pathology of the sperm flagellum: association between flagellar pathology and fertility prognosis in severely asthenozoospermic men. *Hum Reprod* 1998;**13**:2521–2526.

Coetzee K, Kruger TF, Vandendael A, de Villiers A, Lombard CJ. Comparison of two staining and evaluation methods used for computerized human sperm morphology evaluations. *Andrologia* 1997; **29**:133–135.

Coetzee K, Kruger TF, Lombard CJ. Predictive value of normal sperm morphology: a structured literature review. *Hum Reprod Update* 1998; **4**:73–82.

Coetzee K, Kruger TF, Lombard CJ. Repeatability and variance analysis on multiple computer-assisted (IVOS) sperm morphology readings. *Andrologia* 1999a;**31**:163–168.

Coetzee K, de Villiers A, Kruger TF, Lombard CJ. Clinical value of using an automated sperm morphology analyzer (IVOS). *Fertil Steril* 1999b; **71**:222–225.

Coetzee K, Bermes N, Krause W, Menkveld R. Comparison of normal sperm morphology outcomes from two different computer-assisted semen analysis systems. *Andrologia* 2001;**33**:159–163.

Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HWG, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT *et al.* World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 2009; published online: December 4.

David G, Bisson JP, Czyglik F, Jouannet P, Gernigon C. Anomalies morphologiques du spermatozoïde humain. 1. Proposition pour un système de classification. *J Gynecol Obstet Biol Reprod* 1975;**4**:17–36.

Davis RO, Gravance CG. Standardization of specimen preparation, staining, and sampling methods improves automated sperm-head morphometry analysis. *Fertil Steril* 1993;**59**:412–417.

Davis RO, Gravance CG. Consistency of sperm morphology classification methods. *J Androl* 1994;**15**:83–91.

Drevius L-O. Bull spermatozoa as osmometers. *J Reprod Fertil* 1972; **28**:29–39.

Eliasson R. Standards for investigation of human semen. *Andrologie* 1971; **3**:49–64.

Enginsu ME, Dumoulin JCM, Pieters MHEC, Bras M, Evers JLH, Geraedts JPM. Evaluation of human sperm morphology using strict criteria after Diff-Quik staining: correlation of morphology with fertilization in vitro. *Hum Reprod* 1991;**6**:854–858.

Franken DR, Smith M, Menkveld R, Kruger TF, Sekadde-Kigondu C, Mbizvo M, Akande EO. The development of a continuous quality control programme for strict sperm morphology among sub-Saharan African laboratories. *Hum Reprod* 2000;**15**:667–671.

Gago C, Pérez-Sánchez F, Yeung CH, Tablado L, Cooper TG, Soler C. Standardization of sampling and staining methods for the morphometric evaluation of sperm heads in the Cynomolgus Monkey (*Macaca fascicularis*) using computer-assisted image analysis. *Int J Androl* 1998;**21**:169–176.

Gao DY, Ashworth E, Watson PF, Kleinhans FW, Mazur P, Critser JK. Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride, and sucrose on spermolysis. *Biol Reprod* 1993;**49**:112–123.

Garrett C, Liu DY, Baker HWG. Selectivity of the human sperm-zona pellucida binding process to sperm head morphometry. *Fertil Steril* 1997;**67**:362–371.

Hartman CG, Schoenfeld C, Copeland E. Individualism in the semen picture of infertile men. *Fertil Steril* 1964;**15**:231–253.

Henkel R, Schreiber G, Sturmhoefel A, Hipler U, Zermann DH, Menkveld R. Comparison of three staining methods for the morphological evaluation of human spermatozoa. *Fertil Steril* 2008; **89**:449–455.

Hidalgo M, Rodríguez I, Dorado J. Influence of staining and sampling procedures on goat sperm morphometry using the Sperm Class Analyzer. *Theriogenology* 2006;**66**:996–1003.

Hofmann N, Haider SG. Neue ergebnisse morphologischer diagnostik der spermatogeneseströrungen. *Gynäkologe* 1985;**18**:70–80.

Hofmann GE, Santilli BA, Kindig S, Scott RT, Johnson CA. Intraobserver, interobserver variation of sperm critical morphology: comparison of examiner and computer-assisted analysis. *Fertil Steril* 1996; **65**:1021–1025.

Katz DF, Overstreet JW, Samuels SJ, Niswander PW, Bloom TD, Lewis EL. Morphometric analysis of spermatozoa in the assessment of human male fertility. *J Androl* 1986;**7**:203–210.

Kruger TF, Ackerman SB, Simmons KF, Swanson RJ, Brugo SS, Acosta AA. A quick, reliable staining technique for human sperm morphology. *Arch Androl* 1987;**18**:275–277.

Kruger TF, duToit TC, Franken DR, Menkveld R, Lombard CJ. Sperm morphology: assessing the agreement between the manual method (strict criteria) and the sperm morphology analyzer IVOS. *Fertil Steril* 1995;**63**:134–141.

Kruger TF, Lacquet FA, Sanchez Sarmiento CA, Menkveld R, Özgür K, Lombard CJ, Franken DR. A prospective study on the predictive value of normal sperm morphology as evaluated by computer (IVOS). *Fertil Steril* 1996;**66**:285–291.

Lacquet FA, Kruger TF, du Toit TC, Lombard CJ, Sanchez Sarmiento CA, de Villiers A, Coetzee K. Slide preparation and staining procedures for reliable results using computerized morphology. *Arch Androl* 1996; **36**:133–138.

MacLeod J, Gold RZ. The male factor in fertility and infertility. IV. Sperm morphology in fertile and infertile marriage. *Fertil Steril* 1951;**2**:394–414.

Menkveld R. The basic semen analysis. Chapter 9. In: Oehninger S, Kruger TF (eds). *Male Infertility. Diagnosis and treatment.* Oxon, UK: Informa Healthcare, 2007, 141–170.

Menkveld R. Clinical significance of the low normal sperm morphology value as proposed in the 5th WHO laboratory manual for the examination and processing of human semen. *Asian J Androl* 2010; **12**:47–58 (Special edition).

Menkveld R, Kruger TF. Evaluation of sperm morphology by light microscopy. In: Acosta AA, Kruger TF (eds). *Human spermatozoa in assisted reproduction.* Carnforth, England: Parthenon Publishing, 1996, 89–107.

Menkveld R, Stander FSH, Kotze TJW, Kruger TF, van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum Reprod* 1990;**5**:586–592.

Menkveld R, Rhemrev JP, Franken DR, Vermeiden JPW, Kruger TF. Acrosomal morphology as a novel criterion for male fertility diagnosis: relation with acrosin activity, morphology (strict criteria), and fertilization in vitro. *Fertil Steril* 1996;**65**:637–644.

Menkveld R, Lacquet FA, Kruger TF, Lombard CJ, Sanchez Sarmiento CA, de Villiers A. Effects of different staining and washing procedures on the results of human sperm morphology evaluation by manual and computerised methods. *Andrologia* 1997;**29**:1–7.

Menkveld R, El-Garem Y, Schill WB, Henkel R. Relationship between human sperm morphology and acrosomal function. *J Assist Reprod Genet* 2003;**20**:432–438.

Meschede D, Keck C, Zander M, Cooper TG, Yeung CH, Nieschlag E. Influence of three different preparation techniques on the results of human sperm morphology analysis. *Int J Androl* 1993;**16**:362–369.

Mortimer D. *Practical Laboratory Andrology*. Oxford, UK: Oxford University Press, 1994.

Mortimer D, Menkveld R. Sperm morphology assessment—historical perspectives and current opinions. *J Androl* 2001;**22**:192–205.

Ombelet W, Menkveld R, Kruger TF, Steeno O. Sperm morphology assessment: historical review in relation to fertility. *Hum Reprod Update* 1995;**1**:543–557.

Ombelet W, Pollet H, Bosmans E, Vereecken A. Results of a questionnaire on sperm morphology assessment. *Hum Reprod* 1997;**12**:1015–1020.

Oral E, Yetis O, Elibol F, Senol H, Irez T, Aksu FM. Assessment of human sperm morphology by strict criteria: comparison of wet preparation versus stained with the modified Diff-Quik method. *Arch Androl* 2002; **48**:307–314.

Ross KFA. Cell shrinkage caused by fixative and paraffin-wax embedding in ordinary cytological preparations. *Q J Microsc Sci* 1953;**94**:125–139.

Rutlland J, Pommer AC, Meyers SA. Osmotic tolerance limits and properties of rhesus monkey (*Macaca mulatta*) spermatozoa. *J Androl* 2003;**24**:534–541.

Sancho M, Pérez-Sánchez F, Tablado L, de Monserrat JJ, Soler C. Computer assisted morphometric analysis of ram sperm heads: evaluation of different fixative techniques. *Theriogenology* 1998; **50**:27–37.

Soler C, de Monserrat JJ, Gutiérrez R, Nuñez J, Nuñez M, Sancho M, Pérez-Sánchez F, Cooper TG. Use of the Sperm-Class Analyzer® for objective assessment of human sperm morphology. *Int J Androl* 2003; **26**:262–270.

Van der Horst G, Maree L. SpermBlue®: a new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis. *Biotech Histochem* 2009;**84**:299–308.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction*. Singapore: Press Concern, 1980.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction*. Cambridge: Cambridge University Press, 1987. UK.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*. Cambridge, UK: Cambridge University Press, 1999.

World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*. 2010 (in press). http://www.who.int/reproductivehealth/publications/infertility/Examination_and_processing_of_human_semen.pdf.