Article

Motile sperm organelle morphology examination is stricter than Tygerberg criteria

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Abstract

The present study aimed to evaluate the correlation between the motile sperm organelle morphology examination (MSOME) and a well-known sperm morphology classification (Tygerberg criteria). For MSOME, spermatozoa were analysed at ×8400 magnification by inverted microscope equipped with Nomarski differential interference contrast optics, Uplan Apo ×100 oil/1.35 objective lens and variable zoom lens. By Tygerberg criteria, the semen underwent morphological evaluation as described in the literature. Regression analysis demonstrated significant positive correlation between percentage of normal sperm forms by Tygerberg criteria and by MSOME ($r = 0.83$, $P < 0.0001$). However, the incidence of normal spermatozoa by Tygerberg criteria (9.4%) was significantly higher ($P < 0.0001$) than under MSOME (3.3%). Despite the highly positive correlation, MSOME is a much stricter criterion of sperm morphology classification, since it identifies vacuoles and chromatin abnormalities that are not evaluated with the same precision by the analysis of Tygerberg criteria. MSOME should be included among the routine criteria for semen analysis. In addition, MSOME should be used for selection of spermatozoa for intracytoplasmic sperm injection based on the already published literature, as this is a good selection tool.

***Keywords**: MSOME, semen analysis, sperm nuclear vacuoles, Tygerberg criteria*

Introduction

The value of traditional semen analysis has been the object of discussion especially after the introduction of intracytoplasmic sperm injection (ICSI). Via analysis of semen, clinicians expect to obtain a clear indication of fertilization potential, which, except in particular situations (e.g. azoospermia, total teratozoospermia, globozoospermia), conventional evaluation does not provide (Ombelet *et al*., 1997; Menkveld *et al*., 2001). Although none of the semen parameters (or even the functional test), either in isolation or jointly, could be considered definitive, morphology has been shown, consistently, to be the best indicator of male fertility. Diverse studies, originating principally from IVF programmes and intrauterine insemination (IUI), corroborate the sensitivity of morphology as a prognostic factor, particularly for those that utilise the criterion of Tygerberg (strict criteria) (Ombelet *et al*., 1997; Coetzee *et al*., 1998; Gunalp *et al*., 2001; Menkveld *et al*., 2001; Kruger *et al*., 2004; Van der Merwe *et al*., 2005).

On the other hand, different authors observed no relationship between sperm morphology and the success of ICSI (Nagy *et al*., 1998; Oehninger *et al*., 1998; Høst *et al*., 2001). Nevertheless, some studies have shown correlation (Tasdemir *et al*., 1997; De Vos *et al*., 2003; Gvakharia *et al*., 2005). To test the hypothesis that subtle sperm organelle malformations (Berkovitz *et al*., 1999; Bartoov *et al*., 2001) could be associated with the ICSI result, Bartoov *et al*. (2002) developed a new method for real-time evaluation of sperm morphology, the motile sperm organelle morphology examination (MSOME). MSOME is accomplished by utilizing an inverted light microscope equipped with high-power Nomarski optics enhanced by digital imaging to achieve a magnification above ×6000, much higher than the magnification used habitually by embryologists in spermatozoa selection for the ICSI procedure (×200 to ×400) or even that employed in routine semen examination (×1000). Recent studies have demonstrated that intracytoplasmic morphologically

selected sperm injection, based on sperm normality as defined by MSOME, improves ICSI fertilization and pregnancy rates (Bartoov *et al.*, 2003; Berkovitz *et al.*, 2005, 2006a,b; Hazout *et al.*, 2006; Wittemer *et al.*, 2006) and the chance of having a healthy normal child (Berkovitz *et al.*, 2007).

Although MSOME was developed only as a selection criterion, as demonstrated in studies, its application as a method for classifying sperm morphology may represent an improvement in evaluation of semen quality, with potential clinical repercussions, particularly with regard to assisted reproduction techniques. To comprehend the diagnostic/prognostic value, the present study aimed to evaluate the correlation between the MSOME classification and a well-known sperm morphology classification (Tygerberg criteria).

Materials and methods

Study participants

Semen samples (one per subject) were obtained from 97 men from an unselected group of couples undergoing infertility investigation and treatment at the Centre for Human Reproduction Professor Franco Jr.

Sample collection

Semen samples were collected in sterile containers by masturbation after a sexual abstinence period of 2–5 days. A portion of each semen sample was immediately taken and processed for MSOME. The liquefied fresh semen samples were prepared by Isolate (Irvine Scientific, USA) discontinuous concentration gradient. The final pellet was resuspended in 0.2 ml modified human tubal fluid (HTF) medium (Irvine Scientific) and then sent for MSOME.

The remainder of the semen sample was analysed for morphology according to Tygerberg criteria (Menkveld *et al.*, 1990; WHO, 1999) and for standard semen quality parameters according to the World Health Organization (WHO, 1999).

Determination of morphology by MSOME

An aliquot of 1 μl of sperm cell suspension was transferred to a 5μl microdroplet of modified HTF medium containing 8% polyvinylpyrrolidone solution (PVP medium; Irvine Scientific). This microdroplet was placed in a sterile glass dish (FluoroDish; Word Precision Instrument, USA) under sterile paraffin oil (Ovoil-100; VitroLife, Goteborg, Sweden). The sperm cells, suspended in the microdroplet were placed on a microscope stage above an Uplan Apo ×100 oil/1.35 objective lens previously covered by a droplet of immersion oil. In this manner, suspended motile sperm cells in the observation droplet could be examined at high-magnification by the inverted microscope (Eclipse TE 2000 U; Nikon, Japan) equipped with high-power differential interference contrast optics (DIC/Nomarski). The images were captured by a colour video camera containing effective picture elements (pixel) for high quality image production, and a colour video monitor. Morphological evaluation was accomplished on a monitor screen and the total calculated magnification was ×8400.

A spermatozoon was classified as morphologically normal when it exhibited a normal nucleus as well as acrosome, post-acrosomal lamina, neck, tail and mitochondria, besides not presenting a cytoplasmic droplet or cytoplasm around the head (Bartoov *et al.*, 2002). For the nucleus, the morphological state was defined by the form and content of the chromatin. The criterion for normality of nuclear form was a smooth, symmetric and oval configuration. Normal means for length and width were estimated as 4.75 ± 2.8 and 3.28 ± 0.20 μm (Bartoov *et al.*, 2002) respectively, where the form classified as abnormal presented variation of 2 SD in some of the axes (length: ≥5.31 or ≤4.19 μm, width: >3.7 or <2.9 mm). For rapid evaluation of nuclear form, a fixed, transparent, celluloid form of sperm nucleus fitting the criteria was superimposed on the examined cell. In the same manner, the form of the nucleus was considered abnormal if extrusion or invagination of the nuclear chromatin mass had been detected (regional malformation of nuclear form). Chromatin content was considered abnormal if one or more vacuoles were observed to occupy more than 4% of the nuclear area. A nucleus was considered normal if both nuclear form and chromatin content were normal. **Figure 1A** shows normal spermatozoa analysed by MSOME. At least 200 motile spermatozoa per patient were evaluated and the percentage of normal spermatozoa was determined.

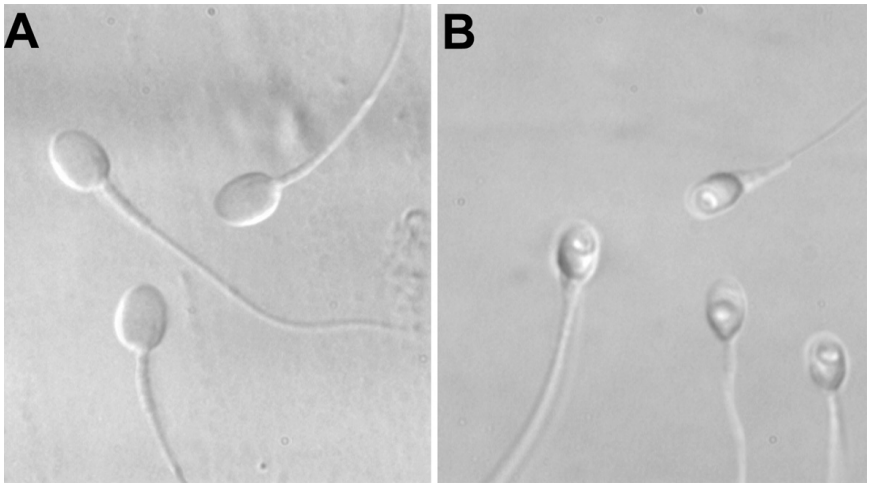

Figure 1. A Normal spermatozoa observed at high magnification (×8400); **B** Spermatozoa with large nuclear vacuoles observed at high magnification (×8400).

Determination of morphology by Tygerberg criteria

The fresh semen underwent morphological evaluation as described in the literature (Menkveld *et al.*, 1990; WHO, 1999). Briefly, 10–20 μl of semen was placed on a pre-cleaned slide and stained using the Papanicolau protocol. At least 200 spermatozoa per patient were evaluated under ×1000 oil immersion magnification and the percentage of normal spermatozoa was determined. For a spermatozoon to be considered normal, the sperm head, neck, midpiece, and tail must be normal. The head should be oval in shape. Allowing for the slight shrinkage that the fixation and staining induce, the length of the head should be 4.0–5.0 μm and the width 2.5–3.5 μm. There should be a well-defined acrosomal region comprising 40–70% of the head area. The midpiece should be slender, 7–8 μm long, less than 1 μm in width and attached axially to the head. The tail should be straight, uniform, thinner than the midpiece, uncoiled and approximately 45–50 μm long. This classification scheme requires that all borderline forms be considered abnormal (Menkveld *et al.*, 1990; WHO, 1999).

Quality control

MSOME

To determine intra-technician variability, fractions of motile spermatozoa were obtained from randomly selected patients. Each sample was observed at least three times by the same observer. A variation of 0.5% was obtained for all parameters analysed: normality of the spermatozoon as a whole, normality of nuclear form, and normality of chromatin.

Tygerberg criteria

Procedures for morphology assessment included a weekly determination of intra-technician coefficient of variation of 1.0% using discarded semen samples. In addition, the stain was checked daily for crossover contamination and changed weekly.

The inter-observer variability was not evaluated because only one observer, blinded to subject identity, performed the entire study (MSOME and Tygerberg criteria).

Statistical analysis

Data were analysed using InStat version 3.0 (GraphPad Software, San Diego, CA, USA) on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA). The Wilcoxon matched-pairs signed-ranks test was used. Correlations were performed using the Spearman rank correlation test. Normal form percentages by Tygerberg criteria and by MSOME were treated as a continuous variable for analysis. The level of significance was set at $P < 0.05$.

Results

The general characteristics of the study population are summarized in **Table 1**. The average age of men was 36.7 ± 6.0 years; 33% had fathered at least one child (or a pregnancy that had ended in miscarriage); 11.3% had varicocoele; 11.3% were smokers; 59.8% made regular use of alcohol and 18.6% regularly took vitamin supplements. The mean duration of infertility was 3.8 ± 3.1 years. Semen analyses showed oligozoospermia (<20 × 10^6 spermatozoa/ml) in 18.6% (18/97) and asthenozoospermia (progressive motility <50%) in 37.1% (36/97) of the patients. According to Tygerberg criteria (Kruger *et al.*, 1986, 1988), 15.5% (15/97) of the semen samples showed ≤4% normal spermatozoa, 69.0% (67/97) showed 5–14% normal spermatozoa and 15.5% (15/97) showed ≥15% normal spermatozoa. Mean values of the other parameters of standard sperm analysis were: ejaculate volume of 3.5 ± 1.3 ml, pH of 8.2 ± 0.32, viability of 60.7 ± 15% and leukocyte number 0.6 ± 0.9 × 10^6.

Regression analysis demonstrated significant positive correlation between percentage of normal sperm forms by Tygerberg criteria and by MSOME ($P < 0.0001$; Spearman's rank correlation coefficient, $r = 0.83$ 95%; confidence interval: 0.75–0.88). **Figure 2** summarizes this result.

In MSOME, the mean incidence of morphologically normal spermatozoa in samples examined was 3.3 ± 3.2% (median: 3%, range: 0–18%). Using Tygerberg criteria, the mean incidence of morphologically normal spermatozoa in samples examined was 9.4 ± 4.8%, (median: 9%, range: 2–23%). The incidence of normal spermatozoa by Tygerberg criteria was significantly higher than under MSOME ($P < 0.0001$), as summarized in **Figure 3**.

The incidence of normal spermatozoa by Tygerberg criteria was also significantly higher than under MSOME in the subgroups of patients with varicocoele (8.7 ± 5.0% versus 2.9 ± 2.7%; $P = 0.0016$), smokers (9.5 ± 4.2% versus 3.1 ± 2.1%; $P = 0.0014$), regular alcohol users (9.2 ± 4.7% versus 3.1 ± 3.3%; $P < 0.0001$) or vitamin supplements takers (10.7 ± 4.7% versus 3.9 ± 3.8%; $P = 0.0002$). Nevertheless, the analysis with two criteria did not yield significant difference in the incidence of normal spermatozoa between individuals who had or did not have varicocoele, smokers or non-smokers, regular alcohol users or non-users, or vitamin supplement takers or non-takers. **Table 2** summarizes these results.

Discussion

The accuracy with which morphological normality of spermatozoa can be assessed depends on the resolution power of the optical magnification system. Spermatozoa appearing as morphologically normal at ×1000 magnification may in fact carry various structural abnormalities that can only be detected at higher optical magnifications (>×6000). The improvement in observation is mainly due to the replacement of Hoffman modulation contrast by the Nomarski interferential modulation contrast. In the present study, it was observed that, despite the strong positive correlation ($r = 0.83$, $P < 0.0001$) found between the two morphological evaluations, the MSOME classification system was shown to be much more restrictive, presenting significantly lower normality percentages ($P < 0.0001$) for the semen samples in comparison to those observed after analysis by the Tygerberg criteria (3 ± 3.2% versus 9.4 ± 4.8% respectively). The MSOME/Tygerberg criteria correlation

Table 1. General characteristics of the study population ($n = 97$).

Characteristic	*Value*
Age (years)	36.7 ± 6.0; 24–56
Duration of infertility (years)	3.8 ± 3.1; 1–16
Fathered at least one child	33 (32)
Mean days abstinence	3.1 ± 1.0
Semen sample[a]	
Volume (ml)	3.5 ± 1.3; 0.4–8.5
pH	8.2 ± 0.3; 8–9
Viability (%)	60.7 ± 15; 8–88
Oligozoospermia	18.6 (18)
Asthenozoospermia	37.1 (36)
Morphology	
≤4% of normal spermatozoa	15.5 (15)
5–14% of normal spermatozoa	69.0 (67)
≥15% of normal spermatozoa	15.5 (15)
Leukocytes ($\times 10^6$)	0.6 ± 0.9; 0–5
Varicocoele	
Yes	11.3 (11)
No	88.7 (86)
Tobacco use	
Yes	11.3 (11)
No	88.7 (86)
Regular alcohol use	
Yes	59.8 (58)
No	40.2 (39)
Vitamin supplement use	
Yes	18.6 (18)
No	81.4 (79)

Unless otherwise stated, values are mean ± SD; range or % (*n*).
[a]Categorized according to World Health Organization and Tygerberg criteria for assessment of morphology (Kruger *et al.*, 1986, 1988; WHO 1999).

appears uninfluenced by varicocoele or the regular use of tobacco, alcohol or vitamin supplements.

Few studies have attempted to analyse MSOME as a morphological classification method for semen. Only Bartoov *et al.* (2002) analysed the relationship between normal spermatozoa obtained by routine analysis (WHO, 1999) and by MSOME in 20 patients. In contrast to the present result, no correlation was found between the frequency of morphologically normal spermatozoa as defined by the WHO and the frequency of normal spermatozoa as defined by MSOME. Nevertheless, as in this study, the incidence of sperm normality by routine analysis (WHO, 1999) was significantly higher than that by MSOME (26.1 ± 7.2%, range 0–68%; and 2.9 ± 0.5%, range 0–5% respectively, $P < 0.01$).

Differences with regard to the observation of nuclear vacuoles can explain the divergence found in normality rates between MSOME and the Tygerberg criteria. The resolution power offered by MSOME (>×6000) enables inclusion of spermatozoa with intranuclear vacuoles that would not be detected in the conventional evaluation. **Figure 1B** shows spermatozoa with large nuclear vacuoles. Bar-Chama *et al.* (2007), employing the Tygerberg criteria, analysed the number of sperm vacuoles in a series of 1295 fresh post-processed sperm samples. They found vacuoles in only 19.5% (253) of the total; 80.5% (1042) had no vacuoles. On the other hand, MSOME revealed that the ejaculates of males routinely referred for ICSI exhibit, on average, 30–40% of spermatozoa with a vacuolated nucleus (Berkovitz *et al.*, 2006b). The mean for spermatozoa presenting nuclear vacuoles in the present study was 80% (>4% of the nuclear area). In addition, there is divergence in quantification, as normal or abnormal, of the presence of nuclear vacuoles. By MSOME, the nuclear chromatin content is considered abnormal if it contained one or more vacuoles that occupied more than

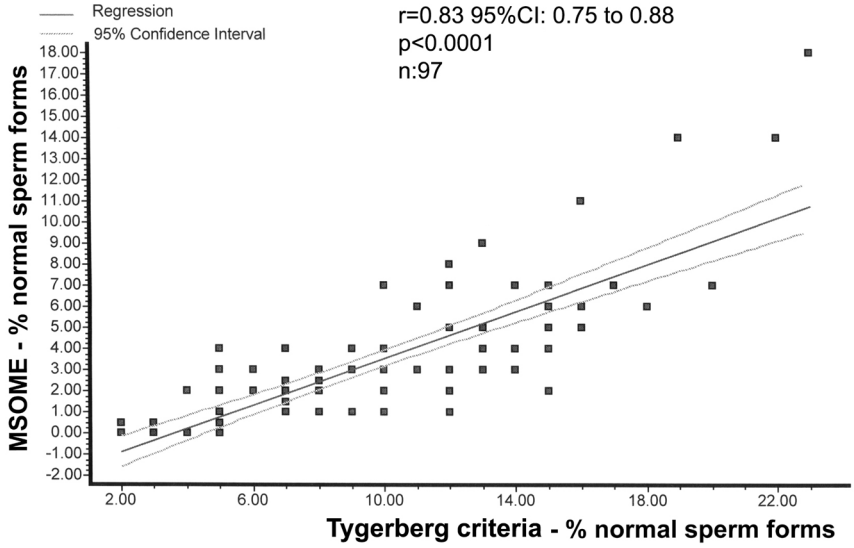

Figure 2. Relationship between percentage of normal sperm forms by Tygerberg criteria and by motile sperm organelle morphology examination (MSOME). Individual data points, regression line and confidence interval (CI) are shown. Spearman rank correlation coefficient = 0.83; $P < 0.0001$.

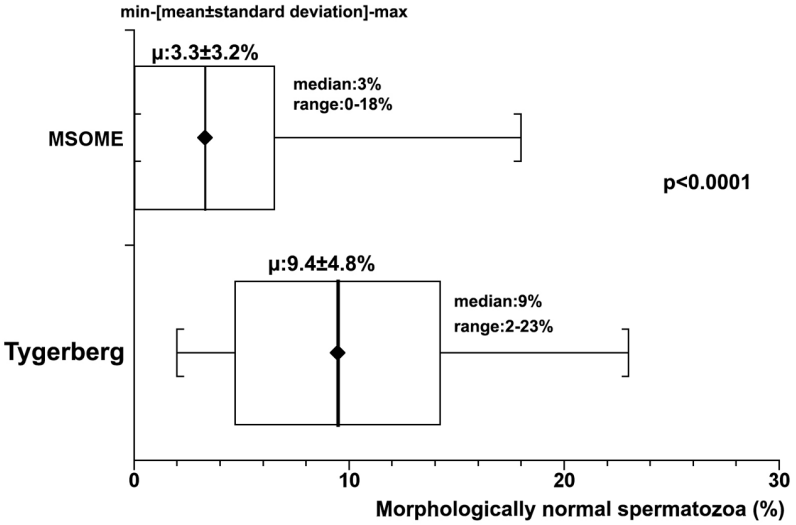

Figure 3. Incidence of morphologically normal spermatozoa according to the two criteria: the incidence of normal sperm by Tygerberg criteria was significantly higher than under motile sperm organelle morphology examination (MSOME) ($P < 0.0001$, Wilcoxon matched-pairs signed-ranks test).

Table 2. Incidence of morphologically normal spermatozoa according to the two criteria for the patient subgroups varicocoele, smokers, regular use of alcohol and vitamin supplement.

Patient subgroup	*Tygerberg criteria normal forms (%)*	*MSOME normal forms (%)*	P-*value*
Varicocele			
Yes	8.7 ± 5.0	2.9 ± 2.7	0.0016
No	9.5 ± 4.8	3.3 ± 3.3	<0.0001
P-value	NS	NS	
Tobacco use			
Yes	9.5 ± 4.2	3.1 ± 2.1	0.0014
No	9.4 ± 4.8	3.3 ± 3.3	<0.0001
P-value	NS	NS	
Regular alcohol use			
Yes	9.2 ± 4.7	3.1 ± 3.3	<0.0001
No	9.8 ± 4.9	3.5 ± 3.1	<0.0001
P-value	NS	NS	
Vitamin supplement use			
Yes	10.7 ± 4.7	3.9 ± 3.8	0.0002
No	9.2 ± 4.8	3.2 ± 3.2	<0.0001
P-value	NS	NS	

MSOME = motile sperm organelle morphology examination; NS = not statistically significant.

4% of the nuclear area (Bartoov *et al.*, 2002). Nevertheless, the Tygerberg criteria are much more tolerant with regard to the presence of vacuoles. A head is considered defective only when >20% of its area is occupied by unstained vacuolar areas (WHO, 1999; Franken and Kruger 2004).

On the other hand, the presence of vacuoles has been defined as deleterious when associated with chromatin lesion. Barth and Oko (1988) and Thundathil *et al.* (1998) reported that nuclear vacuoles in the spermatozoa do not prevent the occurrence of fertilization, but increase the rate of early embryo loss. Bartoov *et al.* (1994) and Mundy *et al.* (1994) observed a clear negative association between the existence of nuclear vacuoles in semen and natural male fertility potential. Berkovitz *et al.*, (2005, 2006a) graded the severity of nuclear morphological alterations, highlighting principally the presence of large vacuoles and suggesting that vacuolization of the sperm nucleus reflects some underlying chromosomal or DNA defects, but did not show data confirming this hypothesis. Berkovitz *et al.* (2006b) and Bach *et al.* (2007) reported that the presence of vacuoles provokes harm to embryonic development, reduction in the pregnancy rate and increase in the miscarriage rate, Franco *at al.* (2008) demonstrated an association between large nuclear vacuoles and both the presence of DNA fragmentation and denaturation

in the spermatozoa. In addition, Garolla *et al*. (2008) showed that the presence of nuclear vacuoles affects mitochondrial function, chromatin status, and aneuploidy rate. Thus, based on clinical/laboratory findings on the repercussions of possible DNA damage for offspring (Carrell, 2008) and considering that sperm nuclear vacuoles are evaluated more precisely by MSOME, the routine employment of morphological sperm evaluation would represent improvement in prognostic terms influencing, consequently, the therapeutic decision, particularly when assisted reproductive techniques are indicated.

Bartoov *et al*. (2002) emphasized that while routine morphological examination is applied in semen samples as a whole, MSOME concentrates only on the fraction of motile spermatozoa. As some morphological defects, such as large vacuoles, can be revealed during sperm movement, motility can thus be an advantage to morphological observation (Berkovitz *et al*. 2005). On the other hand, analysis of only motile spermatozoa by MSOME can signify an additional advantage, since it will provide information on the sample fraction with probable greater real fertilization and development potential. Even though analysis by the Tygerberg criteria employs high magnification, the characteristics of the procedure (fixation and staining) provide no possibility of obtaining information only on the motile portion.

In conclusion, the present results demonstrate that, despite the high positive correlation, MSOME is a much stricter criterion of sperm morphology classification, since it identifies vacuoles and chromatin abnormalities that are not evaluated with the same precision by the analysis of Tygerberg criteria. The MSOME should be included among the routine criteria for semen analysis. In addition, MSOME should be used for ICSI selection of spermatozoa based on the already published literature, as this is a good selection tool.

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