ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

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Keywords:
blastocyst, intracytoplasmic sperm injection, male infertility, motile sperm organelle morphology examination, semen analysis

Received: 5-Dec-2017
Revised: 15-Jan-2018
Accepted: 16-Jan-2018

doi: 10.1111/andr.12473

Sperm morphological normality under high magnification is correlated to male infertility and predicts embryo development

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SUMMARY

Human sperm morphology has been described as an essential parameter for the diagnosis of male infertility and a prognostic indicator of natural or assisted pregnancies. Nevertheless, standard morphological assessment remains a subjective analysis and its impact on intracytoplasmic sperm injection (ICSI) is also of limited value. The objective of this prospective cohort study was to investigate whether motile sperm organelle morphology examination (MSOME) can improve semen analysis by better defining male infertility and providing a better prognosis for ICSI up to a year later. Data were obtained from 483 patients undergoing conventional semen analysis from June 2015 to June 2017 in a private university-affiliated in vitro fertilization (IVF) center. The correlation of MSOME with seminal parameters was evaluated. One hundred and thirty patients underwent ICSI up to a year later, and the correlation between MSOME and ICSI outcomes was established. Except for volume, all seminal parameters were positively correlated with MSOME I+II. MSOME was also distinct between World Health Organization (WHO) classification groups, with normozoospermic and oligoasthenoteratozoospermic presenting the higher and the lower proportion of MSOME I+II, respectively. MSOME I+II was prognostic for fertilization rate, high-quality cleavage-stage embryos rate, and blastocyst rate. The normality cutoff value based on blastocyst rate was MSOME I+II≥ 5.5%. MSOME could be a useful tool for the diagnosis of infertility severity as it is correlated with sperm morphology, motility, and concentration. Men who had higher MSOME I+II had better ICSI outcomes. The future use of MSOME as a routine method for semen analysis may be a reliable form of assessing male infertility.

INTRODUCTION

About 15% of the general reproductive population do not achieve pregnancy within one year of regular sexual intercourse (Boivin *et al.*, 2007; Zegers-Hochschild *et al.*, 2017). Poor semen quality is involved in approximately half of the cases of infertility (Jungwirth *et al.*, 2012; Punab *et al.*, 2016).

Human sperm morphology has been described as an essential parameter for the diagnosis of male infertility and a prognostic indicator of natural (Bonde *et al.*, 1998; Buck Louis *et al.*, 2014; Kovac *et al.*, 2017) or assisted (Kruger *et al.*, 1986; Lundin *et al.*, 1997; Li *et al.*, 2014) pregnancies. Nevertheless, standard morphological assessment of fixed stained cells remains a subjective analysis and is highly dependent on the method, operator, and optical system used. Its impact on intracytoplasmic sperm injection (ICSI) is also of limited value (Nagy *et al.*, 1995; Lundin *et al.*, 1997; De Vos *et al.*, 2003; Ragab *et al.*, 2017).

The real-time morphological evaluation of motile and viable spermatozoa by motile sperm organelle morphology examination (MSOME) is a detailed method that uses high magnification to classify sperm morphology based on the number and size of vacuoles and other abnormalities (Bartoov *et al.*, 2001; Vanderzwalmen *et al.*, 2008). The direct selection of spermatozoa by MSOME for oocyte injection, called intracytoplasmic morphologically selected sperm injection (IMSI), has proved to lead to better ICSI outcomes, such as higher implantation, pregnancy, and live birth rates (Bartoov *et al.*, 2003; Hazout *et al.*, 2006; Setti *et al.*, 2013, 2014a,b).

The World Health Organization has not incorporated the MSOME characterization in the last manual for examination of human semen (WHO, 2010), mostly due to the lack of clarity of the direct correlation of MSOME with male infertility and seminal characteristics, and also because MSOME is still an expensive and time-consuming methodology, so a better

understanding of its real need is necessary. The cutoff values for normal and abnormal seminal parameters defined by WHO are based on sperm morphology, motility, and concentration; different forms of male infertility are categorized as oligozoospermia, asthenozoospermia, teratozoospermia, and the combination of these factors (Cooper *et al.*, 2010).

Although the system for WHO's classification of male infertility would be expected to correlate with clinical outcomes, such as natural or assisted pregnancy rates, reports suggested that the correlation between semen parameters and probability of conception is minimal, if any exists at all. Therefore, the relevance of the WHO classification for treatment prognosis is poor (Esteves *et al.*, 2017), and new classification systems are emerging, such as total motile sperm count (TMSC) (Borges *et al.*, 2016) and the sperm DNA fragmentation (SDF) index (Bungum *et al.*, 2004), to attempt to fill the gap between laboratorial parameters and clinical outcomes.

However, whether the proportion of normal spermatozoa by WHO parameters can be correlated with MSOME classification and whether this classification could be correlated with the outcomes of ICSI cycles are questions that remain to be elucidated. Therefore, the goal of this study was to evaluate whether (i) MSOME classification can bring additional information to semen analysis, in terms of better definition of male infertility; (ii) MSOME classification is a better prognosis to ICSI success compared with standard seminal analysis; and (iii) if it is possible to define an MSOME cutoff value for normal semen based on embryo morphology.

MATERIALS AND METHODS

Experimental design, patients, and inclusion and exclusion criteria

This prospective cohort study included data from 483 patients undergoing conventional seminal analysis for infertility investigation from June 2015 to June 2017, in a private university-affiliated in vitro fertility center. These patients were randomly selected for additional high-magnification morphology characterization by MSOME.

For the investigation of the influence of MSOME classification on ICSI outcomes, the inclusion criteria were as follows: men who had conventional seminal analysis for infertility investigation up to a year before ICSI, and couples undergoing ICSI cycle with fresh embryo transfer performed on Day 5 of development. The exclusion criteria were as follows: couples undergoing ICSI with vitrified/thawed or donated oocytes, surgical sperm retrieval, vitrified/thawed embryo transfer, donated embryo transfer, or pre-implantation genetic diagnosis or screening.

All patients signed a written informed consent form, and the study was approved by the local institutional review board. All laboratorial procedures were performed by the andrology and embryology personnel, which were blinded regarding the study's experiments and groupings.

Semen analysis

All semen samples were collected in the laboratory by masturbation. After liquefaction for 30 min, semen samples were evaluated for sperm count, motility, and morphology. Sperm counting and motility assessment were performed by following the instructions of the counting chamber manufacturer (Leja® slide, Gynotec Malden, Nieuw-Vennep, the Netherlands). The volume of the ejaculate was determined by aspirating the liquefied sample into a graduated disposable pipette. The sperm concentration is expressed as 10^6 spermatozoa/mL, and total sperm count is expressed as 10^6 spermatozoa.

Sperm motility was assessed in 100 random spermatozoa by characterizing them as progressive motility, non-progressive motility, and immotile. The motility was expressed as a percentage. Sperm morphology was evaluated on air-dried smears, fixed, and stained using the quick-stain technique (Diff-Quick; Quick-Panoptic, Amposta, Spain). A total of 200 sperm cells were characterized as morphologically normal or abnormal, and the final morphology was expressed as percentages.

The TMSC was obtained by multiplying the volume of the ejaculate by the sperm concentration and the proportion of progressive motile spermatozoa divided by 100%.

Patients were grouped under normal or abnormal seminal parameters, as defined by WHO (Cooper *et al.*, 2010): normal sperm concentration $\geq 15 \times 10^6$/mL, normal total sperm count $\geq 39 \times 10^6$, normal total sperm motility ≥40%, progressive motility ≥32%, and normal typical morphology ≥4%.

Motile sperm organelle morphology examination (MSOME)

A total of 200 spermatozoa of each raw sample (unprocessed) were analyzed at high magnification using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon, Tokyo, Japan) equipped with high-power differential interference contrast optics (DIC/Nomarski). The total calculated magnification was 6600×. An aliquot of the sperm-cell suspension was transferred to a microdroplet of modified human tubal fluid medium containing 8% polyvinylpyrrolidone (PVP; Irvine Scientific, Santa Ana, CA, USA) in a sterile glass dish (Fluoro Dish; World Precision Instrument, Sarasota, FL, USA). The dish was placed on a microscope stage above an Uplan Apo × 100 oil/1.35 objective lens previously covered by a droplet of immersion oil.

The sperm cells were graded into four groups according to the presence or size of the vacuoles: Grade I, normal form and no vacuoles; Grade II, normal form and ≤2 small vacuoles; Grade III, normal form >2 small vacuoles or at least one large vacuole; and Grade IV, large vacuole and abnormal head shapes or other abnormalities (Vanderzwalmen *et al.*, 2008). The sum of MSOME grades I and II was used as a normal spermatozoa parameter (Berkovitz *et al.*, 2005; Vingris *et al.*, 2015).

Semen preparation

Sperm samples were prepared using a two-layered density gradient centrifugation technique (50% and 90% Isolate, Irvine Scientific, Santa Ana, CA, USA) prior to ICSI.

Controlled ovarian stimulation

Ovarian stimulation was achieved by the administration of recombinant follicle-stimulating hormone (r-FSH, Gonal-F®, Serono, Geneva, Switzerland) and gonadotropin-releasing hormone (GnRH) antagonist, cetrorelix acetate (Cetrotide; Serono Laboratories, Geneva, Switzerland). Ovulation was triggered with recombinant human chorionic gonadotrophin (hCG, Ovidrel™, Serono, Geneva, Switzerland). Oocyte retrieval was performed 35 h later.

Oocyte preparation

Retrieved oocytes were maintained in culture media (Global for fertilization, LifeGlobal, Guilford, CT, USA) supplemented with 10% protein supplement (LGPS, LifeGlobal) and covered with paraffin oil (Paraffin oil P.G., LifeGlobal) for 2–3 h before cumulus cell removal. Surrounding cumulus cells were removed after exposure to a HEPES-buffered medium containing hyaluronidase (80 IU/mL, LifeGlobal). The remaining cumulus cells were then mechanically removed by gently pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, VA, USA).

Oocyte morphology was assessed using an inverted Nikon Diaphot microscope with a Hoffmann modulation contrast system under 400× magnification, just before sperm injection (5 h after retrieval). Oocytes that had released the first polar body were considered mature and were used for ICSI.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection was performed according to Palermo *et al.* (1992), by a highly trained IVF laboratory team. Sperm selection was analyzed at 400× magnification using an inverted Nikon Eclipse TE 300 microscope. The injection was performed in a micro-injection dish prepared with 4-μL droplets of buffered medium (Global w/HEPES, LifeGlobal) and covered with paraffin oil on a heated stage at 37.0 °C ± 0.5 °C on an inverted microscope. Fertilization was confirmed by the presence of two pronuclei and the extrusion of the second polar body approximately 16 h after ICSI.

Embryo quality and embryo transfer

The embryo quality classification was based on criteria determined in an Expert Meeting on Assisted Reproduction, composed of members of the European Society of Human Reproduction and Embryology (ESHRE), held in Istanbul, which defined a globally accepted consensus (Alpha Scientists in Reproductive Medicine and Embryology, 2011). Embryos were morphologically evaluated on days 2, 3, and 5 of development.

To evaluate cleavage-stage morphology, the following parameters were recorded: number of blastomeres, percentage of fragmentation, variation in blastomere symmetry, presence of multinucleation, and defects in the zona pellucida and cytoplasm. High-quality cleavage-stage embryos were defined as those with all of the following characteristics: four cells on day 2, or 8–10 cells on Day 3, <10% fragmentation, symmetric blastomeres, the absence of multinucleation, colorless cytoplasm with moderate granulation and no inclusions, the absence of perivitelline space granularity, and the absence of zona pellucida (ZP) dimorphisms. Embryos lacking any of these characteristics were considered to be of low quality.

To evaluate the blastocyst morphology, embryos were given a numerical score from 1 to 6 based on their degree of expansion and hatching status, as follows: 1, an early blastocyst with a blastocoel that is less than half the volume of the embryo; 2, a blastocyst with a blastocoel that is greater than half the volume of the embryo; 3, a full blastocyst with a blastocoel that completely fills the embryo; 4, an expanded blastocyst; 5, a hatching blastocyst; and 6, a hatched blastocyst The blastocyst rate was defined as the proportion of embryos that reached blastocyst stage (1 to 6) at Day 5 divided by the number of embryos in culture at Day 3 of development.

Embryos were placed in a 50-μL drop of culture medium (Global, LifeGlobal) supplemented with 10% protein supplement and covered with paraffin oil in a humidified atmosphere under 7.5% CO_2 at 37 °C for 3 to 5 days. Embryo transfer was performed on Day 5 of development using a soft catheter with transabdominal ultrasound guidance. One to three embryos were transferred per patient, depending on embryo quality and maternal age.

Clinical follow-up

A pregnancy test was performed 10 days after embryo transfer. All women with a positive test received a transvaginal ultrasound scan after two weeks. A clinical pregnancy was diagnosed when the fetal heartbeat was detected. Implantation rates were calculated per patient. Pregnancy rates were calculated per embryo transfer. Miscarriage rate was calculated per embryo transfer and was defined as a pregnancy loss before 20 weeks.

Data analysis and statistics

Data are expressed as the mean ± standard deviation for continuous variables, while percentages are used for categorical variables. The analysis was performed using SPSS Statistics 20 (IBM, New York, NY, USA).

To assess the association of WHO infertility classification with semen parameters, MSOME morphology classification, and ICSI outcomes, a general linear model (GLM) was used, followed by Tukey's post hoc test. Results are expressed as mean ± standard deviation and *p*-values. The sample size determined was of 305 subjects, using G*Power 3.1.7, considering effect size of 20%, α of 5%, and β of 80%. All the post hoc significances are pointed as different letters.

Linear regression models were used to assess the association of MSOME grades with seminal parameters, adjusted for male age and ejaculatory abstinence, and linear and binary logistic regression models were used to assess the association of MSOME classification with ICSI outcomes adjusted for male age, ejaculatory abstinence, female age, and retrieved oocytes. The sample size determined was 129 subjects, using G*Power 3.1.7, considering effect size of 5%, α of 5%, and β of 80%. The results are expressed as standardized regression coefficients (β) or 95% confidence intervals (CI) of Exp(B), and *p*-values.

Each MSOME grade and combinations of seminal parameters were used to establish the best parameter to discriminate between normal and abnormal semen analysis based on the discrimination of ≥50% blastocyst rate, by receiver operating characteristic (ROC) curve. The best cutoff value was defined by Youden's index.

To cross-validate the prediction generated by ROC curve, a discriminant analysis was performed using as independent variables MSOME I+II, male age, ejaculatory abstinence, female age and retrieved oocytes for the prediction of the grouping variable blastocyst rate, binary defined as <50% blastocyst rate and ≥50% blastocyst rate. Bootstrapping was performed for 1000 samples, and 95% CI defined by bias corrected accelerated.

Generalized estimating equations (GEE) were performed for the comparison of repeated semen analysis of the same individual. Results are expressed as mean ± standard deviation and *p*-values. The coefficient of variation (CV) was calculated as the mean of the divisions between standard deviation and mean of each patient.

RESULTS

During the period covered by this study, 483 men attended our in vitro fertilization center for infertility investigation by conventional seminal analysis and were randomly selected for additional high-magnification morphology characterization by MSOME. The mean age was 37.4 ± 7.1 years old; the ejaculation abstinence was 4.1 ± 2.4 days.

When the WHO criteria were applied, only 38 of the 483 men (7.8%) were classified with normozoospermia (N). Teratozoospermia (T) was the most frequent abnormality, with 335/483 (69.4%) men affected, followed by oligoteratozoospermia (OT) (55/483, 11.4%), asthenoteratozoospermia (AT) (32/483, 6.6%), and oligoasthenoteratozoospermia (OAT) (23/483, 4.8%). None of the patients were diagnosed as oligozoospermia (O) or oligoasthenozoospermia (OA).

The descriptive statistics for semen parameters and morphology characterization by MSOME according to WHO infertility classification is shown in Table 1. The MSOME grades I+II and IV were statistically different between the seminal classifications, with the normozoospermia group having the highest percentage of MSOME grades I+II (14.10 ± 7.01%) and lower percentage of MSOME Grade IV (80.07 ± 5.85%). We observed a decrease in MSOME Grade I+II percentage with the increase in the severity of the seminal classification, and OAT was the most affected group (3.95 ± 3.78%). We did not observe differences in MSOME Grade III proportion between the groups.

The analysis of the direct correlation of MSOME grades with all the isolated seminal parameters is shown in Table 2. Except for volume, all the seminal parameters were positively correlated with MSOME grades I+II ($p < 0.001$) and negatively correlated with MSOME Grade IV ($p < 0.001$). We did not observe correlation of MSOME Grade III proportion with any seminal parameter.

From 483 men under investigation for infertility, 130 (26.9%) returned to our IVF center to perform ICSI up to a year later. The seminal parameters of this group of patients are described in Table 3. The demographic analysis and ICSI outcomes are described in Table 4.

Although the WHO seminal classification had no effect on fertilization rate, high-quality embryos rate at cleavage stage or blastocyst rate (Table 5), we observed a positive correlation of all of the mentioned ICSI outcomes with MSOME grades I+II (Table 6). The high-quality embryos rate at cleavage stage was also positively correlated with the percentage of MSOME Grade III and negatively correlated with MSOME Grade IV.

Table 2 Linear regression analyses of MSOME grades with seminal parameters, adjusted for male age, and ejaculatory abstinence

	MSOME I+II		MSOME III		MSOME IV	
	β	*p*	β	*p*	β	*p*
Volume	−0.031	0.508	−0.029	0.539	0.025	0.592
Concentration	0.281	<0.001	0.022	0.630	−0.252	<0.001
Total sperm count	0.224	<0.001	−0.013	0.782	−0.193	<0.001
Total motility	0.178	<0.001	−0.012	0.791	−0.175	<0.001
Progressive motility	0.192	<0.001	0.008	0.856	−0.188	<0.001
Morphology	0.341	<0.001	0.136	0.003	−0.350	<0.001
TMSC	0.210	<0.001	−0.017	0.716	−0.180	<0.001

β, standardized regression coefficient; MSOME, motile sperm organelle morphology examination; TMSC, total motile sperm count.

Table 3 Descriptive analyses seminal parameters of patients submitted to ICSI

	Mean ± SD
Male age (years)	38.64 ± 7.00
Ejaculatory abstinence (days)	3.99 ± 2.61
Seminal volume (mL)	2.80 ± 1.48
Seminal concentration (× 10^6/mL)	58.78 ± 51.87
Total sperm count (× 10^6)	164.37 ± 171.20
Total sperm motility (%)	58.60 ± 13.74
Progressive sperm motility (%)	49.68 ± 15.76
Sperm morphology (%)	1.37 ± 1.29
TMSC	88.95 ± 101.38
MSOME grades I+II (%)	8.29 ± 6.34
MSOME grade III (%)	5.34 ± 3.97
MSOME grade IV (%)	86.29 ± 8.98

ICSI, intracytoplasmic sperm injection; MSOME, motile sperm organelle morphology examination; SD, standard deviation; TMSC, total motile sperm count.

To define the best MSOME value able to discriminate between normal and abnormal seminal parameters based on blastocyst rate, analyses of ROC curves were performed for individual and combined MSOME grades, and also in combination with others seminal parameters, such as total sperm count, motility, morphology, and TMSC. The highest area under the curve ($AUC_{ROC} = 0.66$) to discriminate between blastocyst rate below and equal or above 50% was obtained with MSOME grades I+II (Fig. 1). The best cutoff value was MSOME I+II ≥ 5.5%, in which a sensitivity of 0.72 and specificity of 0.41 were obtained. The combination with other seminal parameters did not result in increased prediction.

A discriminant analysis was conducted to predict whether ICSI cycle had blastocyst rate of <50% or ≥50%. Predictor

Table 1 Descriptive statistics for semen analysis according to male infertility classification

	N	T	AT	OT	OAT	*p*
Volume (mL)	3.25 ± 1.95[a]	3.14 ± 1.59[b]	4.23 ± 3.11[a]	2.12 ± 1.17[c]	2.13 ± 1.34[c]	<0.001
Concentration (× 10^6/mL)	96.82 ± 47.48[a]	73.33 ± 49.74[b]	39.68 ± 22.74[c]	10.95 ± 12.02[d]	14.12 ± 19.28[d]	<0.001
Total sperm count (× 10^6)	298.9 ± 210.6[a]	214.4 ± 174.7[b]	155.5 ± 128.4[b]	16.69 ± 10.83[c]	17.40 ± 12.94[c]	<0.001
Total motility (%)	66.21 ± 9.84[a]	64.17 ± 10.87[a]	35.75 ± 8.58[c]	57.79 ± 11.03[b]	33.17 ± 9.86[c]	<0.001
Progressive motility (%)	58.05 ± 10.94[a]	55.71 ± 12.43[a]	24.66 ± 4.58[c]	47.44 ± 11.09[b]	21.95 ± 7.54[c]	<0.001
Morphology (%)	4.44 ± 0.64[a]	1.27 ± 1.00[b]	1.00 ± 1.25[c]	0.85 ± 0.93[c]	0.34 ± 0.71[c]	<0.001
TMSC	179.4 ± 143.0[a]	123.3 ± 112.7[b]	38.15 ± 31.14[c]	8.04 ± 5.66[d]	4.21 ± 3.40[d]	<0.001
MSOME I+II (%)	14.10 ± 7.01[a]	9.46 ± 6.33[b]	8.93 ± 16.93[b]	6.92 ± 5.38[c]	3.95 ± 3.78[c]	<0.001
MSOME III (%)	5.81 ± 3.74	5.30 ± 3.87	5.27 ± 6.18	5.18 ± 4.44	4.78 ± 4.12	0.911
MSOME IV (%)	80.07 ± 8.58[a]	85.22 ± 8.41[b]	87.42 ± 12.24[b]	87.74 ± 7.74[b]	91.26 ± 6.81[c]	<0.001

a≠b≠c≠d (GLM post hoc Tukey $p < 0.05$). N, normozoospermia; T, teratozoospermia; AT, asthenoteratozoospermia; OT, oligoteratozoospermia; OAT, oligoasthenoteratozoospermia; TMSC, total motile sperm count; MSOME, motile sperm organelle morphology examination.

Table 4 Descriptive analyses of patient's demographic characteristics and ICSI outcomes

	Mean ± SD
Main Indication (%)	
Male factor	26.9
Ovarian factor	15.4
Tubal factor	5.8
Other female factor	11.6
Mixed factors	31.1
Unexplained	9.2
Female age	36.36 ± 4.04
Total dose of FSH administered (IU)	2375.33 ± 687.41
Number of follicles	14.20 ± 11.92
Number of retrieved oocytes	10.24 ± 9.19
Fertilization rate	86.09 ± 17.58
High-quality embryos rate	40.62 ± 18.10
Blastocyst rate	45.63 ± 35.37
Implantation rate	21.85 ± 36.03
Pregnancy rate (%)	31.6
Miscarriage rate (%)	4.0

ICSI, intracytoplasmic sperm injection; IU, international unit; SD, standard deviation.

variables were MSOME Grade I+II, mother age, retrieved oocytes, father age, and ejaculatory abstinence. The discriminant function revealed a significant association between groups and predictors, accounting for 72.7% of original grouped cases correctly classified, although closer analysis of the structure matrix revealed only two significant predictors, namely MSOME Grade I+II and mother age, with father age, ejaculatory abstinence, and retrieved oocyte being poor predictors. The cross-validated classification showed that overall 63.6% were correctly classified, which is in accordance to was described by AUC_{ROC}.

Using MSOME parameter to define normality, seminal analysis with MSOME I+II < 5.5% had blastocyst rate of 28.53 ± 5.69%, while the rate for MSOME I+II ≥ 5.5% was 50.14 ± 5.05%. This difference was statistically significant ($p = 0.005$) even with the adjustment for male and female ages, ejaculatory abstinence, and retrieved oocytes.

Applying the MSOME normality parameter, 64.5% (312/483) of the samples would be classified as normal, which is eight times more than the normozoospermia percentage obtained with WHO parameters. The MSOME grades I+II normal sample (≥5.5%) includes 92.1% of sperm previously classified as normozoospermia, 69.9% of teratozoospermia, 42.4% of asthenoteratozoospermia, 48.1% of oligoteratozoospermia, and 26.1% of oligoasthenoteratozoospermia. Only 37% of the samples that were abnormal by WHO classification were also abnormal by the MSOME parameter. The descriptive analysis of seminal parameters and ICSI outcomes of normal and abnormal samples by MSOME criteria is shown in Tables 7 and 8, respectively.

Table 6 Linear and binary logistic regression analyses of MSOME grades correlation with ICSI outcomes, adjusted for male and female ages, ejaculatory abstinence, and retrieved oocytes

	MSOME I+II		MSOME III		MSOME IV	
	β	*p*	β	*p*	β	*p*
Fertilization rate	0.197	0.044	0.150	0.134	−0.192	0.052
High-quality embryos rate	0.306	0.013	0.379	0.002	−0.378	0.002
Blastocyst rate	0.248	0.047	0.008	0.954	−0.195	0.130
Implantation rate	−0.098	0.405	−0.137	0.252	0.138	0.244
	95% CI	*p*	95% CI	*p*	95% CI	*p*
Cancelation rate	0.95; 1.07	0.817	0.94; 1.12	0.557	0.95; 1.03	0.716
Pregnancy rate	0.90; 1.05	0.493	0.84;1.09	0.528	0.96; 1.09	0.396

ICSI, intracytoplasmic sperm injection; MSOME, motile sperm organelle morphology examination; β, standardized regression coefficient; 95% CI, 95% confidential interval for Exp(B).

Figure 1 Receiver operating characteristic (ROC) curve of MSOME grades I+II percentage and blastocyst rate (below or equal and above 50%).

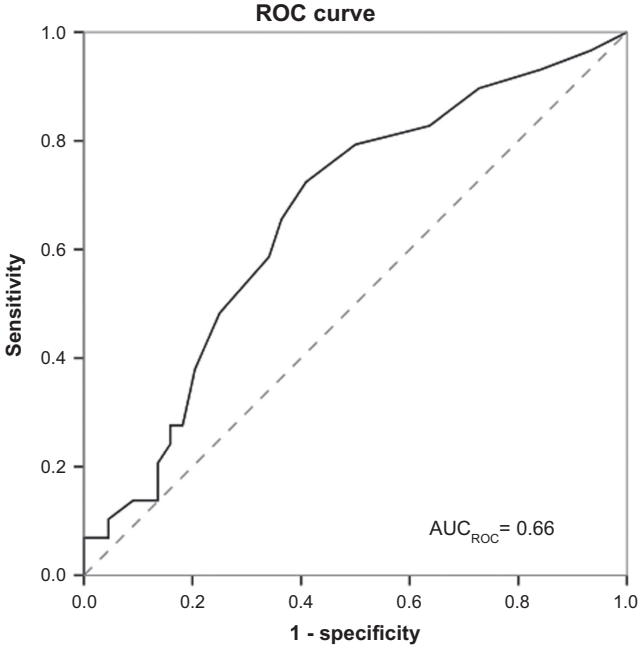

Table 5 Descriptive statistics for the effect of WHO seminal classification on ICSI outcomes, adjusted for male and female ages, ejaculatory abstinence, and retrieved oocytes

	N	T	AT	OT	OAT	*p*
Fertilization rate	86.23 ± 7.30	87.58 ± 2.08	79.12 ± 8.98	83.36 ± 4.67	86.17 ± 6.29	0.846
High-quality embryos rate	47.15 ± 30.12	40.23 ± 17.28	38.75 ± 1.76	35.32 ± 16.31	57.40 ± 25.05	0.414
Blastocyst rate	46.80 ± 32.10	45.14 ± 34.32	26.66 ± 46.18	34.70 ± 38.85	32.58 ± 40.49	0.603
Implantation rate	4.67 ± 20.72	20.32 ± 4.95	50.88 ± 26.35	30.11 ± 14.09	10.46 ± 14.93	0.227
Cancelation rate	55.5%	33.7%	60.0%	47.0%	30.0%	0.433
Pregnancy rate	25.0%	29.3%	50.0%	40.0%	28.6%	0.661

ICSI, intracytoplasmic sperm injection; N, normozoospermia; T, teratozoospermia; AT, asthenoteratozoospermia; OT, oligoteratozoospermia; OAT, oligoasthenoteratozoospermia.

Table 7 Descriptive statistic of seminal parameters per MSOME I+II normality classification

	Normal (MSOME I+II ≥5.5%)	Abnormal (MSOME I+II <5.5%)	*p*
Male age (years)	36.95 ± 6.94	36.88 ± 7.26	0.911
Ejaculatory abstinence (days)	4.22 ± 2.60	3.98 ± 2.16	0.307
Seminal volume (mL)	3.01 ± 1.70	3.00 ± 1.90	0.582
Seminal concentration ($\times 10^6$/mL)	74.46 ± 54.10	42.15 ± 35.65	<0.001
Total sperm count ($\times 10^6$)	215.97 ± 184.55	129.42 ± 152.23	<0.001
Total sperm motility (%)	65.65 ± 12.56	55.92 ± 16.08	<0.001
Progressive sperm motility (%)	53.78 ± 14.02	46.82 ± 17.35	<0.001
Sperm morphology (%)	1.75 ± 1.36	0.82 ± 1.07	<0.001
TMSC	121.93 ± 118.04	69.16 ± 97.11	<0.001
MSOME grades I+II (%)	12.72 ± 7.39	2.90 ± 1.61	<0.001
MSOME grade III (%)	5.99 ± 4.02	3.85 ± 3.35	<0.001
MSOME grade IV (%)	81.27 ± 8.80	93.14 ± 3.76	<0.001

ICSI, intracytoplasmic sperm injection; TMSC, total motile sperm count; MSOME, motile sperm organelle morphology examination.

Table 8 Descriptive statistic of patient's demographic characteristics and ICSI outcomes per MSOME I+II normality classification

	Normal (MSOME I+II ≥5.5%)	Abnormal (MSOME I+II <5.5%)	*p*
Main indication (%)			
Male factor	28.6	33.3	0.383
Ovarian factor	16.0	11.9	
Tubal factor	3.7	4.8	
Other female factor	17.2	4.8	
Mixed factors	22.2	33.3	
Unexplained	12.3	11.9	
Female age (years)	36.18 ± 4.29	36.66 ± 3.58	0.527
Total dose of FSH administered (IU)	2346.38 ± 680.43	2422.61 ± 704.55	0.560
Number of follicles	14.28 ± 12.49	14.06 ± 10.97	0.925
Number of retrieved oocytes	9.92 ± 9.95	10.81 ± 7.74	0.608
Fertilization rate	86.94 ± 19.04	84.59 ± 14.79	0.708
High-quality embryos rate	41.78 ± 16.04	38.40 ± 21.73	0.463
Blastocyst rate	50.14 ± 5.05	28.53 ± 5.69	0.005*
Implantation rate (%)	20.10 ± 35.59	24.24 ± 37.05	0.618
Pregnancy rate (%)	28.26	36.36	0.472

ICSI, intracytoplasmic sperm injection; IU, international unit. *Adjusted for male and female age, ejaculatory abstinence, and number of retrieved oocytes.

Taking into account that semen analyses were performed up to a year before ICSI, there should be long-term stability of MSOME readings for the prediction of blastocyst rate to be consistent. To support the notion of maintained MSOME measurements, male patients that returned for a second semen analysis during the period covered by this study (n = 46) and the seminal parameters of first and second analysis are shown in Table 9. The mean time of return was 133.08 ± 103.91 days, ranging from a minimum of 5 and a maximum of 394 days. There is no statistical difference between any of the semen characteristics, including MSOME measurements. The coefficient of variation (CV) of MSOME grades I+II was 16.5% with a difference of <1 percentage point in the mean between analyses.

Table 9 Long-term stability of MSOME readings of 46 male patients that repeated semen analysis within a year

	First analysis	Second analysis	*p*
Male age (years)	35.87 ± 6.04	36.50 ± 5.62	0.078
Ejaculatory abstinence (days)	4.02 ± 2.13	3.98 ± 1.73	0.895
Seminal volume (mL)	2.90 ± 1.90	2.93 ± 2.00	0.899
Seminal concentration ($\times 10^6$/mL)	62.56 ± 51.75	57.82 ± 61.18	0.459
Total sperm count ($\times 10^6$)	183.81 ± 185.14	172.90 ± 226.83	0.709
Total sperm motility (%)	61.96 ± 16.32	62.65 ± 17.05	0.717
Progressive sperm motility (%)	53.15 ± 17.49	53.54 ± 18.03	0.871
Sperm morphology (%)	1.11 ± 0.97	1.43 ± 1.44	0.175
TMSC	108.34 ± 123.27	99.27 ± 136.81	0.590
MSOME grades I+II (%)	8.69 ± 6.33	9.29 ± 6.85	0.088
MSOME grade III (%)	4.72 ± 3.92	5.39 ± 4.15	0.358
MSOME grade IV (%)	86.67 ± 8.48	85.28 ± 8.96	0.085

ICSI, intracytoplasmic sperm injection; MSOME, motile sperm organelle morphology examination; SD, standard deviation; TMSC, total motile sperm count.

DISCUSSION

There is no consensus as to the optimal MSOME classification cutoff for achieving semen normality and better ICSI outcomes. Therefore, the correlation between MSOME classification and general seminal parameters and its subsequent effects on ICSI outcomes were the aim of this study.

There is a strong correlation between IMSI and better clinical results, mostly in cases of previous ICSI failures, male-factor infertility and advanced maternal age (Setti *et al.*, 2013, 2014a,b). However, the prognostic value of MSOME on routine sperm analysis was to be elucidated. Here, we showed that men who had a higher percentage of MSOME grades I+II spermatozoa on seminal analysis obtained up to one year before ICSI had better outcomes, with higher fertilization rates and increased embryo developmental potential. Considering that the mean percentages of morphologically normal spermatozoa in different MSOME analysis for the same men have a strong correlation up to a year later, as we showed in a group of patients that returned for a second analysis and it is in accordance to what was previously been published (Oliveira *et al.*, 2010), and that no correlation of standard seminal analysis with ICSI outcomes could be observed in the same sample, the use of MSOME as a routine method for semen analysis may be a more reliable form of assessment for male infertility.

MSOME grades I+II together had the stronger predictive values for blastocyst rate. Indeed, the presence of up to two small vacuoles may not directly impact the sperm classification, as we previously described (Vingris *et al.*, 2015) and as others have also proposed (Fortunato *et al.*, 2016), although the direct injection of this class of spermatozoa may impact pregnancy and abortion rates (Berkovitz *et al.*, 2006).

We have previously reported that the increased size and number of vacuoles and abnormalities observed by MSOME have a negative impact on fertilization and blastocyst rates (De Braga Almeida Ferreira *et al.*, 2011; Setti *et al.*, 2014a,b; Vingris *et al.*, 2015) which was confirmed by the present study where poorer semen quality, with higher percentage of MSOME Grade IV, negatively affects ICSI outcomes. The main addition of the present study was that we could establish a connection between semen analysis and ICSI outcomes realized up to one year later, which shows that MSOME has prognostic value. Moreover, we also demonstrated that MSOME has a diagnostic value, as it was correlated with sperm count, motility, and morphology and with male infertility classification groups, in which the poorer the semen quality, the lower the percentage of MSOME grades I+II. These results show that MSOME as a single assay could be used for the diagnosis of infertility severity, and it is directly influenced not only by spermatozoa morphology but also by its motility and concentration.

Our results are also in agreement with other reports that could not find a direct correlation between seminal parameters and ICSI outcomes (Nagy *et al.*, 1995; Gao *et al.*, 2015; Pocate-Cheriet *et al.*, 2017), which reinforces the assertion that MSOME must be incorporated on regular seminal analysis for a better prediction of semen normality and ICSI outcomes. Gao *et al.* (2015) reported that MSOME is a better predictor than other seminal parameters for the outcomes of conventional IVF, even correlating it with pregnancy rate; however, there are substantial differences comparing to our study: they performed MSOME on the day of IVF on processed samples, so there is no long-term prediction estimation and the correlation with raw seminal parameters is poor; and only male patients with normal seminal parameters and female patients with tubal and pelvic factors were included, limiting the external validity of the study.

In an opposite way, Pocate-Cheriet *et al.* (2017) could not find any correlations of MSOME with seminal parameters and ICSI outcomes. This result may be related to the analyzed population of exclusively severe male infertility factors, which had a 10 times higher percentage of MSOME Grade III in comparison with our sample, and may also be a consequence of the lack of adjustment for confounder variables that may introduce a bias, that is, ejaculatory abstinence, male and female age, and cycle outcomes.

Considering the blastocyst rate as a primary outcome to define the normality of the semen sample, we could establish a cutoff value for normality of ≥5.5% of MSOME grades I+II. Applying this parameter, the WHO classification system was demonstrated to be much stricter than the MSOME definition of male infertility, as only 37% of the males with abnormal semen samples as per the WHO (2010) parameters had abnormal MSOME classification.

Samples that had normal seminal parameter applying MSOME classification resulted in around 20% more blastocysts on Day 5. Knowing that embryonic gene expression occurs after the third embryo cleavage, between the 4-cell and the 8-cell stage (Tesarik *et al.*, 1986; Braude *et al.*, 1988), and that paternal genetic heritance directly impacts on the subsequent embryonic development (Tesarik *et al.*, 2004; Tesarik, 2005; Ménézo, 2006), the considerable difference in blastocyst rate among MSOME normal and abnormal groups reflects a straight correlation between high-magnification morphology characterization and sperm function. In discriminant analysis, MSOME I+II was also a significant predictor for blastocyst rate ≥50%.

One could argue that the real effect of MSOME normality classification was to point out cases of pure male infertility; however, our data have the capacity to be generalized to a mixed population, which is what is observed in IVF centers. Additionally, the demographic characteristics (for instance, male and female age, ejaculatory abstinence, FSH administered, number of follicles, and retrieved oocytes) of the MSOME normal and abnormal groups are very similar, with the major differences in seminal parameters.

The limitation of the present study is that we were not able to correlate MSOME normality with implantation and pregnancy outcomes. Although the availability of a higher number of blastocysts for Day 5 embryo transfers is highly correlated with ICSI success (Gardner *et al.*, 2000; Urman *et al.*, 2003; Hill *et al.*, 2013; Kon *et al.*, 2017), embryo implantation does not depend exclusively on proper embryo development, but it also involves the acquisition of a receptive endometrium and its proper dialogue with embryos (Dominguez *et al.*, 2017). Moreover, in a more homogeneous sample the increase in pregnancy rate due to higher MSOME quality had already been reported (Akl *et al.*, 2011; Gao *et al.*, 2015).

In conclusion, the MSOME parameter is more valuable as a predictive tool than the WHO 2010 cutoff values for laboratory results and ICSI outcomes. As these are novel findings for infertile patients undergoing ICSI treatment, prospective randomized studies should be performed to investigate whether the MSOME grading could substitute or add to the WHO classification system for assessing male infertility, and if this single assay could define the outcomes of male-factor-assisted reproduction.

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