ABSTRACT:

The aim of the present prospective study was to determine whether subtle sperm morphological characteristics affect the outcome of intracytoplasmic sperm injection (ICSI), and if so, to identify those that are relevant. For this purpose, we developed a new method, the motile sperm organelle morphology examination (MSOME). The examination is performed in real time using an inverted light microscope equipped with high-power Nomarski optics enhanced by digital imaging to achieve a magnification up to 6300×. MSOME was applied to the leftover sperm fraction selected for microinjection in 100 random couples referred for ICSI treatment at 3 major in vitro fertilization centers. We found that the morphological normalcy of the entire sperm cell, according to MSOME criteria, was positively associated with ICSI fertilization rate (area under the receiver operating characteristics [ROC] curve, 88%) but not with pregnancy outcome. The morphological normalcy of the sperm nucleus, defined by MSOME, was significantly and positively associated with both fertilization rate and pregnancy outcome (areas under the ROC curve, 72% and 74%, respectively). These findings indicate that ICSI-associated pregnancy rate may be affected by subtle morphological malformations of the sperm nucleus, which may remain undetected by the embryologist during the routine selection procedure.

Key words: Motile sperm morphology, ICSI fertilization rate, ICSI pregnancy rate.

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sent prospective study was to assess the relationship between MSOME characteristics and ICSI outcome.

**Materials and Methods**

**Patients**

The study population consisted of the male partners of 100 random couples referred for ICSI treatment at 2 major IVF units in Israel (Rabin and Herzliya Medical Centers; n = 90) or a major IVF unit in France (Laboratoire Marcel Merieux, Institut Rhon-alpin; n = 10). Inclusion criteria included women younger than 37 years and more than 3 retrieved ova per cycle. The mean age of the men was 34.9 ± 4.1 years (range 27–41 years) and of the women, 30.3 ± 5.0 years (range 22–37 years).

**ICSI Procedure**

Indications for ICSI in the 3 IVF centers were as follows: 1) poor (less than 20%) or no (0%) fertilization in the first IVF cycle, 2) immotile spermatozoa, and 3) low percentage of normally formed spermatozoa (less than 5% according to the definition by Kruger et al [1988] or less than 10% according to the World Health Organization [1999]).

At all 3 IVF centers, the motile sperm fractions were prepared for ICSI from ejaculated sperm according to the swim-up method (Le Lannou and Blanchard, 1998). In cases of oligozoospermia (sperm concentration less than 20 × 10⁶ spermatozoa/mL and percentage of motility less than 40%), the density-gradient IxaPrep (Medi-Cult, Copenhagen, Denmark) was used (Yang et al, 1998). Sperm selection for microinjection was performed at a magnification of 200 ×—400 ×; the clinical embryologist excluded spermatozoa from microinjection into the ovum those with severe head-shape defects, such as pin, amorphous, tapered, round, or multinucleated head. After the ICSI procedure was completed, the unused spermatozoa from the motile fractions were transported to the Male Fertility Laboratory at Bar-Ilan University for MSOME.

The microinjection procedure was performed according to the method described by Van Steirteghem et al (1993). Starting on day 21 of the previous cycle or on day 1–3 of the current cycle, patients received gonadotropin-releasing hormone agonist depot or daily leuprorelin (Decapeptyl; Serring, Kiel, Germany) 3.75 mg depot or triptorelin (Decapeptyl) 0.1 mg or intranasal nafarelin (Synarel 200 g; Searle, Wycombe, United Kingdom) 400–800 μg. Pituitary down-regulation was confirmed by a serum estradiol level of less than 50 pg/mL and absence of ovarian cyst (less than 2 cm) on ultrasound scan. When pituitary desen-sitization was achieved, ovarian stimulation was started with hu-man menopausal gonadotropin or human follicle-stimulating hormone. Final oocyte maturation was induced with 10 000 IU human chorionic gonadotropin (hCG) when at least 2 follicles with a mean diameter of more than 18 mm were observed. Oo-cyte retrieval was performed by transvaginal needle-guided ultrasonaspiration at 34 hours after hCG injection.

Fertilization was recorded if 2 pronuclei could be detected after 12 to 16 hours and, finally, if cleavage occurred. A 60% fertilization rate was defined as normal; rates below 60% were defined as low. This cutoff was established prior to onset of the study on the basis of the general IVF-ICSI outcome data obtained from the 3 participating IVF centers. Embryo transfer was usually performed on day 3; on Saturdays and holidays in Israel, it was sometimes performed on day 2. Pregnancy was confirmed at 6 to 7 weeks by ultrasound examination. Pregnancy rate per patient (per cycle) was computed by dividing the general number of pregnancies by the number of patients. Pregnancy rate per transfer was computed by dividing the general number of pregnancies by the general number of embryo transfers. Implantation rate was computed by dividing the number of gestational sacs by the number of transferred embryos.

**Sperm Preparation for Morphological Observation**

The 90 leftover motile sperm fractions from the Israeli IVF units arrived at the Male Fertility Laboratory of Bar-Ilan University within a few hours after microinjection, and the 10 leftover motile sperm fractions from France were sent by special delivery and arrived after 24–48 hours. Because no statistically significant difference was noted in the distribution of the morphological sperm characteristics between the Israeli and French semen samples, all 100 samples were analyzed together.

The fraction of unused spermatozoa, usually suspended in IVF medium (Medi-Cult, Jyllinge, Denmark) or sperm medium (Medi-Cult), was mixed. An aliquot of 1–2 μL of the sperm suspension containing a few thousand spermatozoa was transferred to a microdroplet of sperm medium containing 0%–8% polyvinyl pyrrolidone (PVP) solution (PVP medium 10890001, Medi-Cult) and placed in a sterile Wilco glass-bottomed dish (GWSt-1000; BioSoft International, Amsterdam, The Netherlands) under sterile paraffin oil (OVOL-100; Vitrolife, Göteborg, Sweden). The temperature of the observed sample and the PVP concentration were coordinated with the intensity of the sperm motility. In cases of poor sperm motility, the temperature was elevated to 37°C, no PVP was added to the sperm medium in the recipient droplet, and the sperm suspension medium was supplemented with 6% human serum albumin (Kamada Ltd, Kibbutz Beit Kama, Israel). In cases of high sperm motility, the temperature was lowered to about 20°C and the PVP concentration was kept high (about 8%). Morphological assessment of the sperm cells in motion was made possible by the creation of small bays extruding from the rim of the droplets, which captured the heads of the motile spermatozoa (Figure 1a).

**Sperm Observation**

The leftover motile sperm fraction, prepared for observation, was examined under immersion oil by an inverted microscope (Olympus IX 70) equipped with Nomarski optics, an Uplan Apo X 100/1.35 objective lens, and a 0.55 NA condenser lens. The images were captured by a DXC-950P color video camera (Sony), which has a 1/3-inch, 3-chip power HAD CCD containing some 380 000 effective picture elements (pixels) for high-quality image production, and a color video monitor (Sony PVM-14M4E, HR-Trinitron). The morphological assessment was conducted on the monitor screen which, under the above configuration, reached a real magnification of 6300×, as determined by a 0.01 mm Olympus objective micrometer. Calculation of the total magnification on the television monitor was based on 4 parameters: 1) objective magnification 100×; 2) magnification selector 1.5×; 3) video coupler
Figure 1. High-power light microscope micrograph of motile sperm (1500×). The sperm heads were captured in small bays (BAY) extruding from the rim of the droplets. N indicates morphologically normal spermatozoa; LV, large nuclear vacuoles; SV, small nuclear vacuoles; LO, large oval sperm cells; Nr, spermatozoa with a narrow postacrosomal region; PA, partial acrosome.

Table 1. Specific morphological malformations of the sperm cell subcellular organelles observed by MSOME

<table>
<thead>
<tr>
<th>Sperm Subcellular Organelles</th>
<th>Specific Malformations</th>
<th>Chromatin Content</th>
<th>Neck</th>
<th>Tail</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrosome</td>
<td>Lack</td>
<td>Vacuolar area &gt;4% of the whole nuclear area</td>
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<tr>
<td></td>
<td>Partial vesiculated</td>
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<td></td>
<td>Vesiculated</td>
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<td></td>
<td>Small oval</td>
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<tr>
<td></td>
<td>Large oval</td>
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</tr>
<tr>
<td></td>
<td>Narrow (&lt;2.9 μm in width)</td>
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<tr>
<td></td>
<td>Wide (&gt;3.7 μm in width)</td>
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<td></td>
<td>Short (&lt;4.2 μm in length)</td>
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<tr>
<td></td>
<td>Regional disorder</td>
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<td></td>
<td>Abaxial disorder</td>
<td></td>
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<td></td>
<td>Cytoplasmic droplet</td>
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<tr>
<td></td>
<td>Lack</td>
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</tr>
<tr>
<td></td>
<td>Partial disorganization</td>
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</tbody>
</table>

* Sperm cells with pin, round, amorphous, tapered (cigar-shape), or multinucleated heads were not assessed in this study.

It should be stressed that MSOME was applied exclusively to motile spermatozoa, which under low light microscopy magnification, have a high potential to be selected for ICSI. Sperm cells with a severe malformation, such as a pin, amorphous, tapered, round, or multinucleated head, which can be clearly identified even by low magnification (200×–400×), were not assessed in this study, because such spermatozoa are routinely eliminated from injection into the oocyte.

One-hundred motile spermatozoa from each sperm sample were examined for the morphological state of 6 subcellular organelles: acrosome, postacrosomal lamina, neck, mitochondria, tail, and nucleus. The first 5 of these subcellular organelles were considered morphologically normal or abnormal on the basis of the presence of specific malformations (presented in Table 1), which were defined according to the arbitrary descriptive approach adopted in our previous studies for sperm examination by transmission and scanning electron microscopy (Bartoov et
al., 1981; Glazerman and Bartoo, 1993). For the nucleus, the morphological state was defined by both shape and chromatin content. The criteria for a normally shaped nucleus by MSOME, as by electron microscopy, were smooth, symmetric, and oval configurations. The average lengths and widths of this configuration was estimated in 100 spermatozoa with a normal nucleus and were found to be 4.75 ± 0.28 μm and 3.28 ± 0.20 μm, respectively. For rapid evaluation of the nuclear shape, a fixed, transparent, celluloid form of a sperm nucleus fitting these criteria was superimposed on the examined cell; the nuclear shape was documented as abnormal if it veered in length or width by 2 standard deviations from the normal mean axes values (Figure 1a and b). An extrusion or invagination of the nuclear chromatin mass was defined as a regional nuclear shape malformation (Table 1). The nuclear chromatin content was considered abnormal if it contained one or more vacuoles that occupied more than 4% of the normal nuclear area (Figure 1a and b). To be considered morphologically normal, a sperm nucleus had to have both a normal shape and a normal chromatin content. A sperm cell exhibiting a normal nucleus as well as a normal acrosome, postacrosomal lamina, neck, tail, mitochondria, and no cytoplasmic droplet or cytoplasm around the head was classified as morphologically normal (Figure 1a and b).

The relationship between normal spermatozoa obtained by the routine method (World Health Organization, 1999) and by MSOME was assessed in 20 of the 100 examined patients. No significant correlation was found between the frequency of morphologically normal spermatozoa as defined by the World Health Organization and the frequency of morphologically normal spermatozoa as defined by MSOME. It is noteworthy that routine morphological examination is applied to the entire semen sample, whereas MSOME concentrates only on the motile sperm fraction. The incidence of sperm normalcy by routine sperm analysis was significantly higher than that by MSOME (26.1% ± 7.2%, range 0%–68%; and 2.9% ± 0.5%, range 0%–5%, respectively, F = 38.2, P < .01).

**MSOME Quality Control**

To determine intratechnician variability in the assessment of sperm morphology by MSOME, motile sperm cell fractions were obtained from 5 randomly selected patients who had been referred to our laboratory for this procedure. Each sample was observed 5 times (total, 5 × 5 observations) by the same observer (BB), who was blinded to the source of the 25 samples, and were found to be 4.75 ± 0.28 μm and 3.28 ± 0.20 μm, respectively. For rapid evaluation of the nuclear shape, a fixed, transparent, celluloid form of a sperm nucleus fitting these criteria was superimposed on the examined cell; the nuclear shape was documented as abnormal if it veered in length or width by 2 standard deviations from the normal mean axes values (Figure 1a and b). An extrusion or invagination of the nuclear chromatin mass was defined as a regional nuclear shape malformation (Table 1). The nuclear chromatin content was considered abnormal if it contained one or more vacuoles that occupied more than 4% of the normal nuclear area (Figure 1a and b). To be considered morphologically normal, a sperm nucleus had to have both a normal shape and a normal chromatin content. A sperm cell exhibiting a normal nucleus as well as a normal acrosome, postacrosomal lamina, neck, tail, mitochondria, and no cytoplasmic droplet or cytoplasm around the head was classified as morphologically normal (Figure 1a and b).

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**Results**

During this study a total of 1074 oocytes was retrieved. Sperm injection was performed in 908 mature oocytes (85%), and fertilization occurred in 591 (65%). The mean fertilization rate per case was 64.2% ± 2.2% (median 62.4%; Figure 2). No statistically significant correlation was observed between the fertilization rate following ICSI and the number of retrieved or injected ova, or between the fertilization rate and the age of the male or female partners.

On MSOME, the mean incidence of morphologically normal spermatozoa in the samples examined was 3.3% ± 0.5% (median 2.5%, range 0%–38%). Only one man exhibited the extreme value of 38% for this MSOME parameter; in the other 99 patients, the incidence was 0%–15%. The incidence of morphologically normal spermatozoa was found to have a positive and significant correlation with the fertilization rate following ICSI (r = 0.52, P < .01; Figure 3a); this correlation held true even when the patient with 38% normal spermatozoa was excluded. Division of the patients by fertilization rate (Table 2) showed that the subgroup with a low fertilization rate (<60%, n = 47) exhibited a significantly lower incidence of morphologically normal spermatozoa than the subgroup with a normal fertilization rate (>60%, n = 53; 1.3% ± 0.3% vs 5.8% ± 0.6%, F = 28.2, P < .01). None of the 24 men with 0% morphologically normal spermatozoa in the sperm motile fraction exhibited a normal fertilization rate (mean 38.7% ± 2.3%; Figure 3a). Using ROC curve analysis, we demonstrated that the incidence of spermatozoa with normal morphology observed by MSOME had a very high predictive value for fertilization rate following ICSI (area under the ROC curve, 88%; cutoff value, 2.5%; optimal sensitivity, 81%; optimal specificity, 78%; Table 3; Figure 4a).

Of the 6 sperm subcellular organelles examined by our correlation analysis was used to identify relationships between MSOME characteristics and ICSI outcome, and analysis of variance was used to compare sperm samples with decreased and normal fertilization rates and samples from men who did and did not achieve pregnancy. Nonparametric statistics—the Kruskal-Wallis test followed by the Mann-Whitney U test—were performed concomitantly to avoid possible artifacts caused by the high standard deviations of some of the sperm parameters.

ROC curve analysis was used to determine the prognostic accuracy of each MSOME characteristic as well as its ability to correctly classify subjects into decreased and normal fertilization subgroups and successful and failed pregnancy subgroups. The sperm morphological parameters were analyzed for diagnostic sensitivity and specificity, and the threshold for each analyzed parameter was calculated for optimal sensitivity and specificity. The area under the ROC curve was expressed as a percentage.
method, only the morphological normalcy of the sperm nucleus (shape + chromatin content) correlated significantly with fertilization rate ($r = 0.42, P ≤ .01$; Figure 3b). The mean incidence of this sperm parameter was $26.8\% \pm 1.7\%$ (median 25.0\%, range 0\%–68\%). It was able to significantly differentiate the normal from the low fertilization subgroup ($F = 11.2, P ≤ .01$; Table 2), and had predictive value for the ICSI fertilization rate (area under ROC curve, 72%; cutoff value, 24.5%; optimal sensitivity, 66%; optimal specificity, 64%; Table 3; Figure 4a). None of the statistical methods revealed any significant association between ICSI fertilization rate and the other MSOME parameters detailed in Table 1.

Both the parametric and the nonparametric methods agreed completely; that is, the morphological integrity of the entire sperm cell as well as of the sperm nucleus, as defined by MSOME, were associated with IVF-ICSI outcome parameters, whereas the other sperm morphological characteristics examined in this study had no effect on ICSI outcome.

Twenty-eight pregnancies (28\%) were achieved during this study. In 5 cases, no embryos were transferred; therefore, the mean pregnancy rate per transfer was $29.5\% \pm 1.6\%$. No significant correlation was observed between the achievement of pregnancy and any of the following parameters: number of retrieved oocytes, number of injected ova, fertilization rate, age of the man, or age of the woman. There was also no statistically significant correlation between pregnancy rate and the incidence of morphologically normal spermatozoa observed by MSOME. Of the 6 sperm subcellular organelles examined, only the morphological normalcy of the sperm nucleus (shape + chromatin content) correlated significantly with achievement of pregnancy ($r = 0.38, P ≤ .01$). A comparison of the patients who did and did not achieve pregnancy revealed that this sperm parameter was able to significantly
differentiate between the successful and failed subgroups (n = 28 and n = 67, respectively; \( F = 13.1, \ P \leq .01 \); Table 2). It is noteworthy that no patient who exhibited less than 20% spermatozoa with a morphologically normal nucleus achieved pregnancy (Figure 5). ROC curve analysis demonstrated that normalcy of the sperm nucleus had predictive value for pregnancy occurrence (area under the ROC curve, 74%; cutoff value, 28.5%; optimal sensitivity, 68%; optimal specificity, 67%; Table 3; Figure 4b). None of the statistical methods revealed any significant association between pregnancy rate and the other MSOME parameters detailed in Table 1.

**Discussion**

The assessment of the possible association between male fertility potential and sperm morphology in humans is very complicated, mainly for two reasons: human spermatozoa exhibit a high structural variability, and clinicians cannot be sure that the sperm cells randomly selected for examination indeed represent those that are capable of fertilizing the ova. The first problem can be solved by morphological classification of the many specific sperm cell malformations according to the separate subcellular organelles, as shown in our earlier study (Bartoov et al, 1981). The morphological state of the sperm organelles arbitrarily defined by this method was found to have the highest predictive ability for male fertility potential (Bartoov et al, 1990, 1994). The second difficulty can be solved in the IVF-ICSI era by morphologically examining the single spermatozoa selected for microinjection. The new MSOME approach combines both these solutions. It enables a morphological analysis at the subcellular level of single motile sperm cells in real time.

We are aware that the application of MSOME to a spermatozoon microinjected into an ovum would have provided very clear answers regarding the relationship between MSOME characteristics and ICSI outcome. However, the embryologist could not ethically select impaired sperm cells when morphologically normal spermatozoa were present. Therefore, for the present study, we used 100 random sperm cells from the leftover fraction rather than the actual spermatozoon selected for microinjection. Although this compromise probably decreased the significance of the association, our results nevertheless demonstrate that the morphological state of the entire sperm cell, and especially of its nucleus, has predictive value for fertilization, pregnancy outcome, or both following ICSI. The present results disagree with the current, widely accepted opinion that neither the type nor the extent of sperm impairment has an important influence on the outcome of ICSI (Mansour et al, 1995; Nagy et al, 1995, 1998; Svalander et al, 1996; Kupker et al, 1998; Oehninger et al, 1998).

According to the present study, spermatozoa free of any specific morphological malformations exhibited the highest positive correlation with ICSI fertilization rate. This finding supports the studies of Mercan et al (1998) and Osawa et al (1999) who reported slightly reduced fertilization rates in cases of a low incidence of morphologically normal spermatozoa. The significant association of the normalcy of the sperm cell with the fertilization rate, but not with the achievement of pregnancy, may be explained by the very narrow range of distribution (0%–15%) of this parameter, making it insufficiently sensitive...
Figure 4. ROC curve analysis. (a) Predictive values of morphologically normal spermatozoa (———) and spermatozoa with morphologically normal nucleus (———) for ICSI fertilization rate. (b) Predictive value of spermatozoa with morphologically normal nucleus for achieving pregnancy following ICSI.

Figure 5. Distribution of spermatozoa with morphologically normal nucleus in the successful and failed pregnancy subgroups (AV, average value ± SEM).

to differentiate the failed pregnancy subgroup from the successful one, which numbered only 28 patients. By contrast, the normalcy of the sperm nucleus exhibited a very wide range (0%–68%). Therefore, this sperm parameter is in high correlation with both ICSI fertilization and pregnancy outcome.

Of the 6 sperm subcellular organelles examined, a morphologically normal sperm nucleus proved to be the most important sperm parameter influencing ICSI outcome. This finding supports some investigations showing low pregnancy and implantation rates in cases of total sperm head teratozoospermia (Tasdemir et al, 1997) and low fertilization rates in specific cases of globozoospermia (Liu et al, 1995; Battaglia et al, 1997) and megalozoospermia (Kahraman et al, 1999). The existence of association between nuclear normalcy, defined by MSOME, and ICSI outcome confirms our previous electron microscopy study showing the importance of the normalcy of the sperm head subcellular organelles in achieving pregnancy by both IVF and ICSI (Berkovitz et al, 1999). We assume that normalcy of the sperm nucleus, as observed by MSOME, reflects nuclear DNA content and organization, which have been reported to exert a significant effect on ICSI fertilization rate and embryo development (Sun et al, 1997; Lopes et al, 1998; Sakkas et al, 1998; Obasaju et al, 1999).

Given that the normalcy of the sperm nucleus is indeed strongly correlated with ICSI outcome, if the embryologist is unable to identify the fine normalcy of the sperm nucleus—which can be done naturally by the zona pellucida—why is the pregnancy rate following classical IVF not superior to that for ICSI? It is possible that the ability of the ICSI process to overcome sperm tail abnormalities (Berkovitz et al, 1999) balances the inability of the embryologist to select spermatozoa with the most appropriate nucleus.

The relatively low area under the ROC curve and low optimal sensitivity and specificity values for the normal nucleus in predicting ICSI pregnancy rate (72%, 68%, and 67%, respectively) may have been due to our investigational approach, as discussed above. In addition, the wide, random ICSI population selected for this prospective study probably included patients with egg factor or other female factor infertility. At the same time, the finding of a significant correlation between the nuclear morphological normalcy and the ICSI pregnancy rate despite these shortcomings warrants the initiation of a clinical study involving the exclusive selection of spermatozoa with normal nuclei, as defined by MSOME, prior to microinjection.

In conclusion, the MSOME method developed in our laboratory revealed that the morphological normalcy of the sperm nucleus is an important factor for achieving pregnancy following ICSI. This finding suggests that ICSI outcome may be improved by the exclusive microinjection into the oocyte of spermatozoa with a normal nucleus. This assumption requires validation by further clinical studies.

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