Motile Sperm Organelle Morphology Examination (MSOME)

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Introduction

The application of assisted reproduction techniques has provided help to many men seeking to father a child, although the current success of these procedures remains suboptimal. For many years the sperm selection methods were based on washing procedures with subsequent resuspension of the male germ cells. Double density gradient centrifugation and the swim-up procedure were used as standard preparations. Today some protocols allow sperm to be selected according to their ultrastructural morphology.

On the other hand, successful human reproduction relies partly on the inherent integrity of sperm DNA. Clinical evidence has shown that sperm nuclear DNA damage is closely related to male-derived repeated failure of ICSI attempts [1, 2]. It was also noted that the late paternal effect, but not the early one, is associated with increased sperm DNA fragmentation [2]. Sperm DNA damage is associated with a significantly increased risk of pregnancy loss after IVF and ICSI [3, 4].

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Therefore, it is now necessary to improve the safety of the sperm selection method. It is urgent to optimize procedures to isolate spermatozoa for ICSI with low risk of DNA damage. In recent years, one technology has attracted the attention of specialists as a method capable of identifying a spermatozoon with low risk of DNA damage: ultrastructural morphology sperm selection at high magnification [5, 6].

Motile Sperm Organelle Morphology Examination (MSOME)

The accuracy with which morphological normality of spermatozoa for ICSI can be assessed depends on the resolution power of the optical magnification system. Conventionally, ICSI is performed with a ×20/×40 objective, resulting in an overall optical magnification of 200–400× [7, 8] (Fig. 8.1). At this magnification, only major sperm morphological defects can be observed, whereas it is more difficult to identify subtle sperm organelle malformations that seem to be related to the ICSI outcome. To test this latter hypothesis, in 2001, Bartoov's group developed a new method of unstained, real-time, high-magnification motile sperm organellar morphology examination called MSOME. High magnification is made possible by the use of an inverted light microscope equipped with high-power Nomarski differential interference contrast optics enhanced by digital imaging to achieve a

A. Agarwal et al. (eds.), *Non-Invasive Sperm Selection for In Vitro Fertilization: Novel Concepts and Methods*,
DOI 10.1007/978-1-4939-1411-1_8,

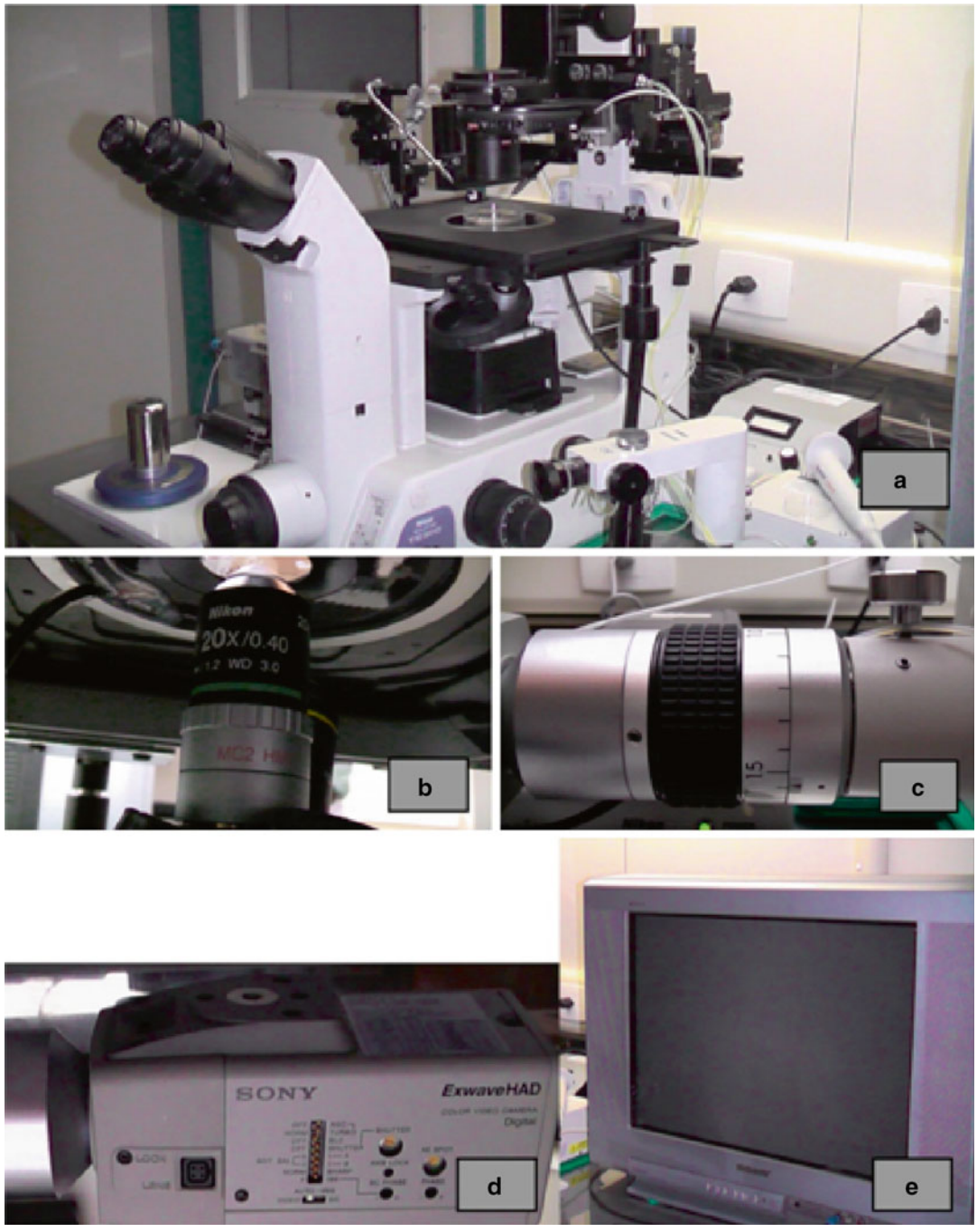

Fig. 8.1 (**a**) The polarization inverted microscope (TE 300 Nikon, Japan) equipped with Hoffman contrast and polarizing lens; (**b**) Hoffman objective of 20×; (**c**) C-mount; (**d**) Camera; and (**e**) 21-inch monitor

magnification of up to 6,600× [5, 6]. Inclusion of MSOME, together with a micromanipulation system, enables the retrieval of a single motile spermatozoon with strictly defined morphologically normal nucleus to be injected into the retrieved oocytes. Bartoov and his group named this modified IVF procedure intracytoplasmic morphologically selected sperm injection (IMSI) [5].

Furthermore, spermatozoa appearing morphologically normal at this magnification (×400) may in fact carry various structural abnormalities that can only be detected at higher optical magnification. The spermatozoa with vacuoles would not be detected in conventional ICSI [7]. This is a serious disadvantage, because microinjection of spermatozoa with vacuolated nuclei has been shown to be associated with low implantation and pregnancy rates, and with early miscarriage [9, 10].

Sperm Preparation and Microscopy Equipment for MSOME

In our laboratory, a 1 µl aliquot of sperm cell suspension was transferred to a 5 µl microdroplet of modified HTF medium containing 7 % 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 an inverted microscope (Eclipse TE 2000 U; Nikon, Japan) equipped with high-power differential interference contrast optics (DIC/Nomarski). The images were captured by a color video camera containing effective picture elements (pixel) for high-quality image production, and a color video monitor (Fig. 8.2). Morphological evaluation was accomplished on a monitor screen and the total calculated magnification 8,400× (total magnification: objective magnification = 100 × magnification selector = 1.0 × video coupler magnification = 1.0 × calculated video magnification = 84.50) [11, 12].

MSOME Criteria for Selected Sperm Cells

Normal Spermatozoa

A spermatozoon was classified as morphologically normal (Fig. 8.3a) when it exhibited a normal nucleus as well as acrosome, post-acrosomal

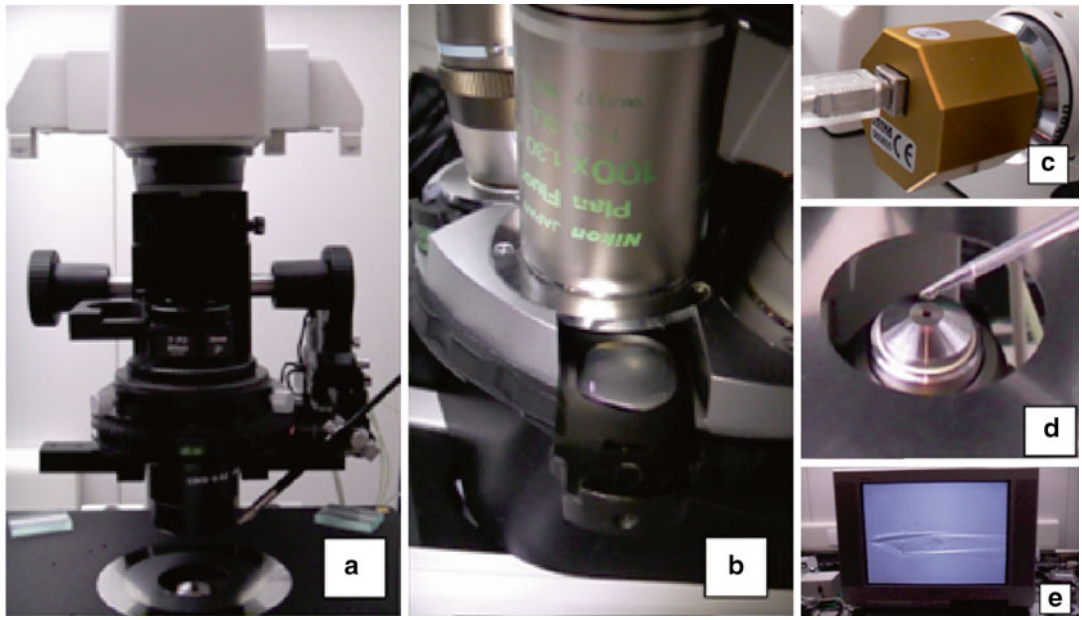

Fig. 8.2 Microscope equipment for MSOME: (**a**) DIC system; (**b**) Plan Fluor Apo ×100 oil/1.35 lens + Prism; (**c**) Color video camera; (**d**) Immersion oil on objective; (**e**) Video monitor 21″

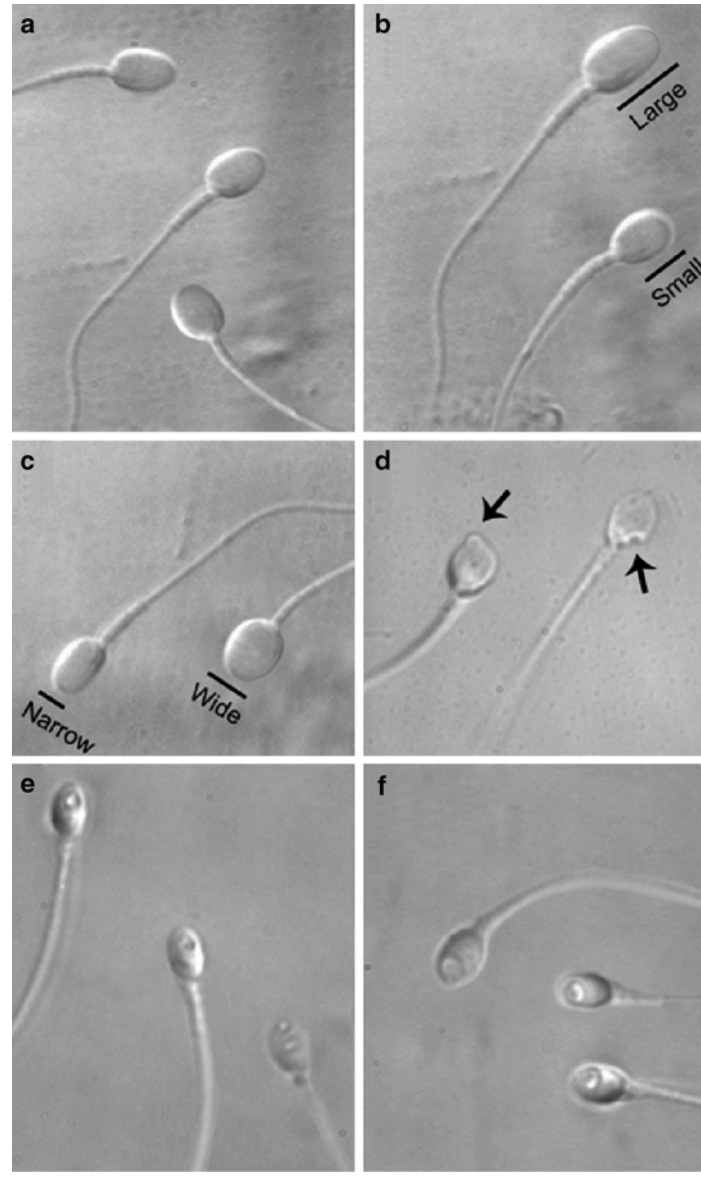

Fig. 8.3 (**a**) Normal spermatozoa, (**b**, **c**); Abnormality of nuclear form: spermatozoa with small or large oval nuclear forms (**b**); spermatozoa with wide or narrow nuclear forms (**c**); (**d**–**f**) Abnormalities in nuclear chromatin: spermatozoa with regional shape abnormality of nuclear form (**d**), spermatozoa with vacuoles occupying 5–50 % of the nuclear area (**e**), spermatozoa with large nuclear vacuoles (>50 % of the nuclear area) (**f**)

lamina, neck, and tail, besides not presenting a cytoplasmic droplet or cytoplasm around the head [5, 6]. 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, respectively, whereas the form classified as abnormal presented variation of 2SD in at least one 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 should be superimposed on examined cell (chablon construction based on ASTM E 1951-2). In the same manner, the form of the nucleus was considered normal if no extrusion or invagination of the nuclear chromatin mass had been detected (regional abnormality of nuclear form). Chromatin content was considered normal if the total vacuole area was found to occupy less than 4 % of the nuclear area. A nucleus was considered normal if both nuclear form and chromatin content were normal.

Abnormalities of Nuclear Form

(a) Spermatozoa with small or large oval nuclear forms (Fig. 8.3b). Sperm cells exhibiting an abnormal but oval nuclear shape and a morphologically normal nucleus, content length ≤4.19 or ≥5.31 μm.
(b) Spermatozoa with wide or narrow nuclear forms (Fig. 8.3c). Sperm cells with non-oval, abnormal nuclear shapes, but with normal nuclear content, width: >3.7 or <2.9 mm.
(c) Spermatozoa with regional shape abnormality of nuclear form (Fig. 8.3d). Sperm cells with an extrusion or invagination of the nuclear mass.

Abnormalities of Nuclear Chromatin Content

(a) Spermatozoa with vacuoles occupying >4–50 % of the nuclear area (Fig. 8.3e).
(b) Spermatozoa with large nuclear vacuoles (Fig. 8.3f). sperm cells with vacuoles occupying >50 % of the nuclear area.

Sperm cells with a severe abnormality (such as pin, amorphous, tapered, round, multinucleated head, double tail) easily identified at low magnification (200–400×) were not assessed. The abnormalities observed at high magnification, in both form and nuclear content, also presented normal acrosome, post-acrosomal lamina, neck, and tail, and did not show a cytoplasmic droplet or cytoplasm around the head. Spermatozoids that presented more than one alteration were classified as having the most severe alteration (small/large < wide/narrow < regional shape abnormality < with vacuoles occupying >4–50 % < with vacuoles occupying > 50 % of the nuclear area).

MSOME and Sperm Nuclear Vacuoles

One specific sperm malformation, which has been negatively associated with natural male fertility potential, is the presence of large nuclear vacuoles. In 2006, Berkovitz et al. [9] carried out a more specific analysis on the impact of sperm cells with normal nuclear shape but large vacuoles, identified by MSOME, on ICSI pregnancy outcome. They performed a comparative study testing the outcomes of two matched IMSI groups: an experimental group ($n=28$), where spermatozoa with strictly defined normal nuclear shape but large vacuoles were available for oocyte microinjection, and a control group ($n=28$), where strictly defined morphologically normal spermatozoa (including nuclear shape and content) were retrieved for microinjection into the oocytes. Both groups satisfied the following selection criteria: maternal age <40 years and three or more retrieved metaphase II oocytes in the present cycle. As a result, the groups showed no differences as to fertilization, implantation rates, or development of top quality embryos, whereas the pregnancy rate per cycle in the experimental IMSI groups was significantly lower, and the early miscarriage rate per pregnancy was significantly higher than that of the control group (18 % versus 50 %, $p<0.01$, and 80 % versus 7 %, respectively, $p<0.01$). In this work, therefore, retrieval of spermatozoa with strictly normal nuclear shape but large vacuoles appeared to reduce ICSI pregnancy outcome and to be associated with early miscarriage. In fact, embryo development seemed normal at the early stages (no differences in top quality embryos, normal fertilization, or implantation), whereas it seemed impaired at the later ones (low pregnancy and high miscarriage rates).

On the other hand, the rate of vacuolated spermatozoa increases with the patient's age, regardless of vacuole size (occupying less or more than 4 % of the sperm head area) [13]. The rate of spermatozoa containing large vacuoles (≥50 % of sperm head area) also increases with patient's age [14]. The vacuole area increased significantly when semen parameters were impaired. These impairments included a decrease in sperm concentration, altered vitality, and a reduced number of spermatozoa with normal morphology [15].

There is evidence that DNA damage may derive from abnormal chromatin packaging due to underprotamination, which induces DNA

strand breaks [16, 17]. In the same way, Franco Jr et al. [18] showed that the presence of large nuclear vacuoles reflects the presence of abnormal chromatin packaging, which may facilitate sperm DNA damage.

At the level of the sperm cell, the presence of large sperm head vacuoles can be considered a potential indicator of sperm nuclear abnormalities (chromatin descondensation). At the level of the male population, these vacuoles relate particular male infertility factors (age, abnormal chromatin compaction, increased DNA fragmentation, and abnormal conventional semen parameters). However, the evaluation of sperm head vacuoles in daily practice remains nondefinitely standardized, with varying methods being used [15, 19].

In addition, according to Berkovitz et al. [20] sperm nucleus morphological normality, assessed at high magnification, could decrease the prevalence of major fetal malformations in ICSI offspring. Recently, Cassuto et al. [21] compared the risk of major malformations of children born after standard ICSI and after IMSI, a prospective population-based study was conducted from 2005 to 2010. ICSI and IMSI were performed in only one assisted reproduction unit. Medical data and follow up during 2 years of 1028 infants were collected. Major malformations were identified and classified by an external independent physician. The two groups were similar concerning the parent's age, treatment, number of oocytes recovered, days of transfer, gestational age, and birthweight. However, major malformations were significantly lower with IMSI (4/450, 1.33 %) versus ICSI (22.578, 3.80 %) (adjusted odds ratio 2.84, 95 % confidential interval 0.14–0.87, $p=0.014$).

MSOME and Embryo Development

Recently, it has been demonstrated that IMSI has no significant effect on embryo quality at day 2 in relation to the conventional ICSI procedure [22]. In addition, a relationship has been shown between defective spermatozoa and higher early miscarriage rates, despite the apparent lack of decrease in embryo quality on day 3 [10]. Based on the hypothesis that the employment of spermatozoa with large nuclear vacuoles would not produce any early paternal effects on embryo development (up to day 3), Vanderzwalmen and his group [23] investigated the possible influence of such nuclear vacuoles on the embryo's competence to develop to the blastocyst stage; according to the researchers this may suggest a late paternal influence on embryo development after paternal DNA content begins to contribute to such advancement at around day 3 after fertilization. The outcome of embryo development (until day 5) was assessed in a group of 25 patients who underwent sibling oocyte microinjection with four different grades of sperm cells: (1) grade I (absence of vacuoles); (2) grade II ($\leq$2 small vacuoles); (3) grade III (>2 small vacuoles or $\geq$1 large vacuole); and (4) grade IV (large vacuoles with abnormal head shape or other abnormalities). Small (<4 % of the head volume) and large vacuoles were defined according the classification of Bartoov [5, 6], while grading was performed between 6,000× and 12,000× high magnification. To reduce the influence of female factor infertility, the inclusion criteria were female age less than 40 years and availability of at least eight oocytes at retrieval. As a whole, the four groups did not differ significantly as to the number of zygotes and embryo development up to day 3, including the subgroup analysis; on the contrary, the data showed highly significant differences in the development to blastocysts and the blastocyst quality among the four grades ($p<0.001$). On the other hand, comparing the groups one by one with regard to development to blastocysts, statistically significant differences were found between groups I (56.3 %) and II (61.4 %) and between groups III (5.1 %) and IV (0 %). Even when combining the groups into pairs, no significant difference in terms of embryo development to day 3 was seen (group I/II: 87.1 % versus group III/IV: 66.7 %), whereas the incidence of blastocyst and good quality blastocyst formation was significantly different between combined grades I/II and grades III/IV spermatozoa (43.5 and 19.1 % versus 10.1 and 2.9 % respectively, $p<0.001$). Based on these

results, the researchers postulated that the size and number of sperm nuclear vacuoles, identified accurately at high magnification, negatively affected blastocyst development, especially after a day-3 embryo transfer, and reinforced previous studies suggesting the paternal effects on initial embryonic development [23, 24].

MSOME for Routine Laboratory Semen Analysis

Although MSOME was initially developed by its creator only as a selection criterion [5], its application as a morphological semen classification method could represent an improvement in routine laboratory sperm analysis, with potential clinical implications, especially in the field of medically assisted reproduction. In the past, only one study [6] has examined the relationship between normal spermatozoa obtained by the WHO routine method [25] and by MSOME in 20 patients. As a result, no significant correlation was found between the incidence of morphologically normal spermatozoa as defined by the WHO and by MSOME. Nevertheless, routine analysis reported a significantly higher percentage of sperm normality in relation to MSOME. However, Oliveira et al. [26] adopted a similar approach, evaluating the correlation between MSOME classification and a highly diffuse sperm morphology classification (Tygerberg criteria) [27], in order to better understand the potential diagnostic/prognostic value of the MSOME method. The study design included 97 randomly selected semen samples. Regression analysis showed a significant positive correlation between the incidence of normal sperm forms by Tygerberg criteria and that obtained by MSOME ($r = 0.83$; 95 % CI: 0.75–0.88; $p < 0.0001$). Similarly to the work presented by Bartoov et al. [6], the MSOME criteria appear to be much more restrictive, presenting significantly lower sperm normality percentages for the semen samples in comparison to those found after routine analysis by the Tygerberg classification (3.3 ± 3.2 %, range 0–18 %; and 9.4 ± 4.8 %, range 2–23 % respectively, $p < 0.0001$). Based on these results, the researchers postulated that despite the high positive correlation, MSOME represented a much stricter evaluation criterion for sperm morphology, since its resolution power (≥6,000×) enabled the identification of vacuoles and chromatin abnormalities that could not be described with the same accuracy by a Tygerberg method analysis. In addition, its focus on motile sperm fractions could only represent an additional advantage for MSOME by providing information on the fertilization and development potential of the sample fraction referred for assisted reproduction treatment. Therefore, our group stressed the importance of not only including MSOME among the criteria for routine laboratory semen analysis, but of performing this step prior to the conventional ICSI procedure [26].

In 2006, Hazout et al. [10] reported a positive association between high-magnification selection of sperm cells with normal nuclear shape and pregnancy outcome in patients with repeated conventional ICSI failures; in a subgroup of patients ($n = 72$) involved in the study, the level of sperm DNA integrity (by TUNEL assay) was assessed, and the outcomes of IMSI could be compared in patients with several degrees of sperm DNA damage. The improvement of clinical ICSI outcomes was evident both in patients with an elevated degree of sperm DNA fragmentation and in those with normal sperm DNA status.

In 2008, Franco et al. [11] compared the amount of DNA fragmentation (by TUNEL assay) and the incidence of denatured single-stranded or normal double-stranded DNA (by acridine orange fluorescence method) in sperm cells characterized by the presence of large nuclear vacuoles (LNV group) and in strictly morphologically normal spermatozoa (NN group), both selected at high magnification (8,400×). The analyses were carried out on fresh semen samples of 30 unselected patients. The authors reported a significantly higher level of DNA fragmentation in LNV sperm cells than in NN spermatozoa (29.1 % versus 15.9 %, $p < 0.0001$). In addition, the LNV group also showed a significantly increased amount of single-stranded denatured DNA with respect to

the NN sperm cells (67.9 % versus 33.1 %, $p<0.0001$). Thus, we postulated that the high levels of denatured DNA in LNV sperm cells pointed to early decondensation and disaggregation of sperm chromatin fibers: an unwanted high degree of sperm decondensation could result in asynchronous chromosome decondensation, and may lead to cytoplasmic fragments in the embryo [28]. Therefore, the data presented stressed the link between the presence of large nuclear vacuoles and increased DNA damage in sperm cells and supported the routine selection of morphologically motile spermatozoa at high magnification (by MSOME) before conventional ICSI. A similar approach was adopted by Garolla et al. [29] in particular, after observation via a high magnification system (×13,000), 20 strictly morphologically normal sperm cells (for acrosome, head, neck, and tail, including those cells which had large nuclear vacuoles, but were otherwise normal) were selected from each semen sample of ten patients with severe testicular alteration and absent sperm motility: ten with normal morphology and no vacuoles (group A) and ten with normal morphology and at least one large head vacuole (group B), for a total of 200 sperm cells. Each spermatozoon was studied for mitochondrial membrane potential, DNA integrity (by acridine orange staining), DNA fragmentation (by TUNEL assay), and sperm aneuploidies (by FISH analysis). The data showed that single cells from group A exhibited a significantly better physiological status than cells from group B with regard to mitochondrial function, DNA integrity, and DNA fragmentation (13.3 ± 4.9 % versus 52.2 ± 14.7 %, 5.3 ± 3.0 % versus 71.9 ± 11.1 %, and 9.3 ± 4.8 % versus 40.1 ± 11.6 % respectively, all $p<0.001$). No chromosomal alteration was present in cells from group A. Therefore, although the study was conducted on a highly restricted number of cells, the results strengthened the concept of an association between the incidence of sperm DNA damage and the presence of nuclear vacuoles and stressed the importance of a morphological selection by high-magnification microscopy [24].

In conclusion, all the publications [7, 9, 10, 30–32] about IMSI reported not only better results as to implantation and clinical pregnancy rates but also a reduction in the miscarriage rates in couples whose sperm cells were strictly morphologically selected at high magnification (MSOME). In addition, the IMSI procedure improved the clinical ICSI outcome in patients with several degrees of sperm DNA damage.

It has been reported that the presence of vacuoles could reflect molecular anomalies responsible for abnormal chromatin remodeling during the sperm maturation process and may contribute to making the spermatozoa more susceptible to sperm DNA impairment. The current results establish a crucial association between normal blastocyst development and both the number and size of vacuoles, indicating that routine morphological analysis of sperm cells, performed at high magnification (6,000×), is of fundamental importance to improving the embryo implantation potential. In addition, the presence of vacuoles in the nuclei of spermatozoa is also associated with reduced pregnancy and with a high level of DNA damage.

Conclusions

Spermatozoa appearing morphologically normal at a magnification of ×400 may in fact carry various structural abnormalities. With the advent of MSOME it is now possible to analyze the spermatozoa under high-magnification, which allows the detection of nuclear vacuoles that may affect the ICSI outcomes. In light of the findings, the MSOME method seems to be a powerful tool for selecting strictly morphologically normal spermatozoa, with a lower incidence of DNA defects that cannot be detected at routine ICSI magnification.

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