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Sperm

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Importance of Spermatozoa Morphology in Fertility

The introduction of intracytoplasmic sperm injection (ICSI) more than 20 years ago was a tremendously helpful tool to overcome the infertility of couples when conventional *in vitro* fertilization (IVF), partial zona dissection, or subzonal sperm injection treatments had failed [1]. The injection of a single spermatozoon into the ooplasm allowed an adequate answer to severe male infertility diagnosis indicated by low sperm count, poor sperm motility, or both or to infertility due to morphology deficiency. Interestingly, since the introduction of ICSI, less attention has been devoted to the sperm's morphology itself. In addition, it is even more remarkable that after the introduction of ICSI, even though human spermatozoa exhibit a wide range of shapes, several studies found no correlation between the injection of sperm with normal or abnormal morphology and ICSI outcomes [2,3]. However, such observations were most probably biased by the selection performed by the embryologist who tried to select the best "normal-looking" motile spermatozoa before ICSI, which does not always reflect the quality of the whole semen population.

The assessment of sperm morphology by Kruger's strict criteria is routinely applied and widely accepted as the best method of prediction for male fertility potential and highlights the concept that sperm morphology is a very important parameter in the analysis of the whole population of a semen sample [4]. Of all semen parameters, sperm morphology turns out to be the best predictor of a man's fertilizing potential [5].

But the situation is completely different when we move from the diagnostic level toward the laboratory practice of ICSI where we have to select only one spermatozoon for injection.

Although there is still a controversy as to whether morphological defects of spermatozoa even have an effect on fertilization or subsequent embryonic development [6], it becomes more and more accepted that abnormally shaped spermatozoa from patients diagnosed with terato- and asthenozoospermia have a significantly increased frequency of aneuploidy, a higher DNA fragmentation index (DFI), and an increased rate of mitochondrial dysfunction [7]. The importance of morphologically normal sperm selection is reinforced when facing the reproductive outcomes in terms of fertilization, embryo development, pregnancy rates, and abortions rates when the oocyte injections can only be done with abnormally shaped spermatozoa, that is, sperm with elongated, tapered, or amorphous heads; broken necks; or cytoplasmic droplets [8].

In view of ICSI bypassing the natural barriers of reproduction, it seems reasonable to develop optimized sperm selection techniques.

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With the implementation of restrictive laws regulating the number of embryos to transfer, methods of gamete and embryo selection are of paramount importance. If we keep in mind that ICSI bypasses the natural barriers of reproduction and that an abnormal spermatozoon bears the danger of transferring putative negative effects on to the offspring, it might be reasonable to develop optimized selection techniques. Now, for almost a decade, therefore, more attention has been directed toward physiological selection methods based on the biochemical ability of the spermatozoa to bind either to solid hyaluronic acid or to zona pellucida before intracytoplasmic injection [9].

In addition, the importance of sperm morphology is being studied [8], with several novel microscopic approaches, such as differential interferential contrast microscopy [10], digital holographic microscopy [11], and atomic force microscopy [12], currently being used or in development to allow a more detailed observation of the different parts of the sperm head (nucleus and acrosome), midpiece, and tail.

Bartoov et al. [10] introduced, already 10 years ago now, an innovative, noninvasive technique for a more precise morphological evaluation of motile spermatozoa. The so-called motile-sperm organelle-morphology examination (MSOME) changed the perception of how a spermatozoon suitable for injection should appear. In fact, MSOME permits not only simple observation in terms of a sperm's size and shape but also a detailed examination in real time of the subtle subcellular morphology and abnormalities, such as nuclear defects. The MSOME technique allowed even a more strict discrimination of spermatozoa, with normal nuclei defined by an oval shape with a smooth configuration including a normal nuclear content [10,13].

With the replacement of the standard bright-field or the Hoffman modulation contrast (HMC) optics by the Nomarski differential interference contrast (DIC) optics, a better three-dimensional view of the sperm head became available. Bright-field or HMC can reveal major abnormalities of the spermatozoon's aberrations in head shape and at the level of the midpiece and tail. With Nomarski interference contrast, a more in-depth evaluation of the quality of semen that provides substantial information about the normalcy of the head, neck, and midpiece is possible. In consequence, this method of spermatozoa evaluation and selection for ICSI indication generated the intracytoplasmic morphologically selected sperm injection (IMSI) technique [14,15].

The aim of the first part of this chapter is to give the reasons to select spermatozoa free of nuclear and neck–midpiece defects or those from patients suffering from globozoospermia syndrome. In addition, a brief overview of the putative nature of nuclear vacuoles according to the present status of knowledge and their consequences on embryo development is presented. In the second part of this chapter, we depict the different spermatozoa classification systems and the technical procedures to select spermatozoa so as not to impair oocyte quality.

Tangible Arguments to Select Sperm Free of Defects

**One decade after the first application of MSOME and IMSI, can we still consider these as useful techniques?**

Sperm Head Vacuoles: An Additional Sperm Defect That Can Be Better Highlighted with Nomarski Optics

The meticulous approach in sperm selection applying the Nomarski DIC optics allows the detection of subtle sperm head nuclear abnormalities that were designated as vacuoles by Bartoov et al. [10] (Figure 4.1a). These nuclear defects are otherwise not evident at a 400× magnification with HMC microscopy [15–17] (Figure 4.1b).

In fact, we had to wait for the introduction of Nomarski optics to be concerned about the presence of large and small vacuoles and then consider them as potential defects. Before the introduction of MSOME and IMSI, respectively, there was to our knowledge no report of the presence of vacuoles and an analysis of their location, origin, structure, and effect on reproductive outcome after the conventional ICSI procedure.

Spermatozoa might contain heterogeneous vacuoles varying in number, size, and content. To simplify this matter, we often only refer to the term "vacuole" in any circumstance. But is vacuole the appropriate terminology? From a biological point of view, vacuoles are vesicles surrounded by a membrane. However, this structural abnormality that we airy term vacuole might more likely represent a kind of depression in the nuclear part of the head. Some of these so-called vacuoles are in fact deep, like a crater or hollow. Watanabe et al. [18] suggested that vacuoles are hollows whereby the plasma membrane falls into the nucleus, and Westbrook et al. [19] even talked about nuclear crater formation in the sperm head. Toshimori [20] differentiates between large vacuoles with amorphous substances or membranous structures inside and small, vacuolous patterns without any structures inside.

Boitrelle et al. [12,21] reported that the plasma membrane was sunken, but intact and large and small vacuoles were identified as an abnormal, "thumbprint"-like nuclear concavity covered by acrosomal and plasma membranes.
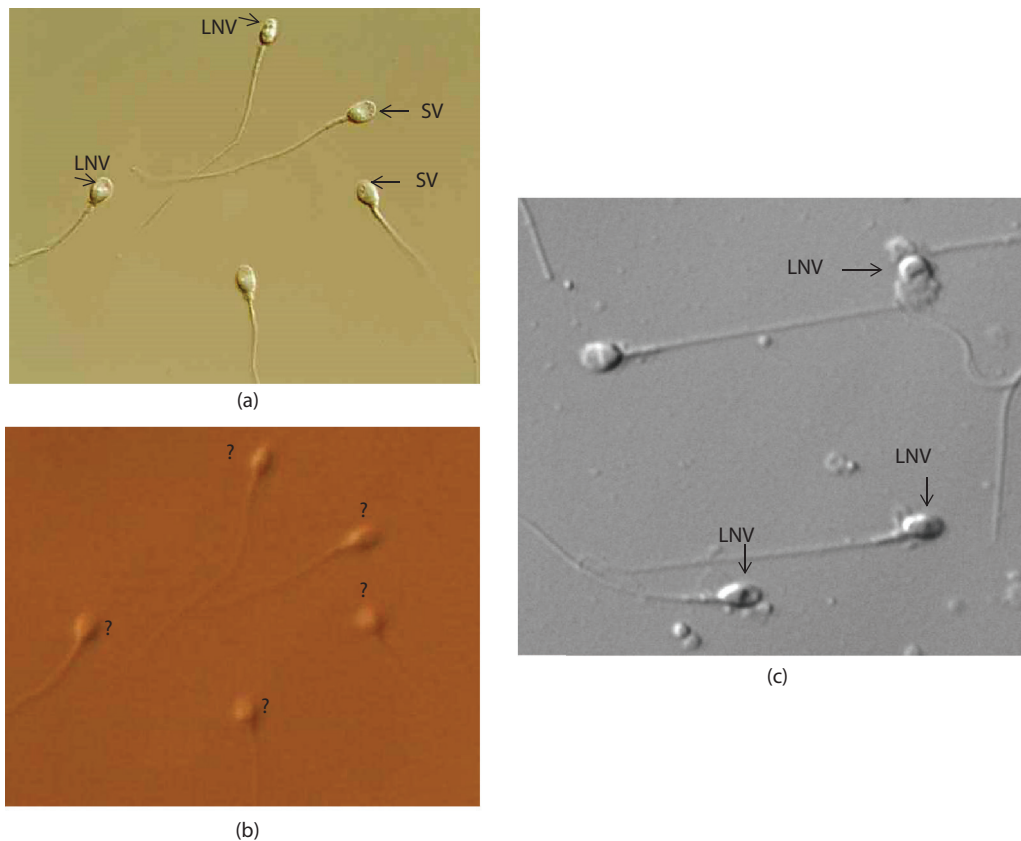
FIGURE 4.1 Observation of spermatozoa with HMC and DIC microscopy (Nomarski). (a) Nomarski at 6000× magnification. (b) Conventional HMC at 6000× magnification. (c) Conventional HMC at 600× after adjustment of the polarizer. SV, small vacuole; LNV, large nuclear vacuole.

Reasons to Select Vacuole-Free Sperm

Pathological Character of Nuclear Vacuoles

One crucial question to investigate concerns the significance of vacuoles. Vacuoles are like liquid bubbles that can be seen in the sperm's head, where the nucleus is located, and that have been correlated with DNA anomalies. Considering the publications of Berkovitz et al. [15], Cayli et al. [22], and Hazout et al. [23], it is most likely that vacuoles reflect molecular defects that are responsible for anomalies of sperm chromatin packaging and abnormal chromatin remodeling during sperm maturation; such defects, in turn, may render spermatozoa more vulnerable to DNA damage. According to different studies, the integrity of the chromatin is related to the presence or absence of vacuoles in the head of spermatozoa and the loss of chromatin compaction renders the exposed DNA more prone to reactive oxygen species [24–26].

Several DNA and chromatin staining assays, including aniline blue, chromomycin A3, and acridine orange, were applied to assess more precisely the integrity of the DNA in vacuolated spermatozoa. Interestingly, several studies found highly statistically significant differences in the DNA integrity between spermatozoa with or without vacuoles [27].

According to the growing body of literature on this subject, it is now more and more admitted that the presence of large nuclear vacuoles is correlated with failures of chromatin condensation [7,28,29]. It is reported in selected teratozoospermic populations that sperm vacuoles were exclusively of nuclear origin and preferentially located

to the anterior part of the sperm head [30]. Chromatin condensation defects were the main alterations observed in spermatozoa with large vacuoles [7,12,21,28,30–32]. The chromatin with large vacuoles was atypically decondensed, showing a high degree of immaturity.

Indeed, chromatin condensation is a crucial step in protecting the paternal genome during the transit from the male to the oocyte before fertilization (epididymal transit) [33]. Rousseaux et al. [34] demonstrated a new keystone in DNA compaction in humans and murines. They postulated that histones are replaced by transitional proteins called bromodomains before protamination takes place. Structural abnormalities of the nucleus include incomplete or impaired chromatin condensation and nuclear vacuoles and inclusions. Karyolytic changes or the presence of large intranuclear lacunae or vacuoles are the morphological manifestations of underlying biochemical alterations.

Early embryogenesis is a critical time for epigenetic regulation, and these epigenetic modulation processes are sensitive to environmental factors [35,36]. Epigenetic patterns are usually faithfully maintained during development. However, this maintenance sometimes fails, resulting in the disturbance of epigenetic processes and representing the basis of numerous human disorders. It is known that before histone replacement by protamines, the nucleosomes are destabilized by hyperacetylation and DNA methylation levels rise [37,38]. These potential epigenetic mechanisms could be implied in chromatin condensation failures [39].

Poor chromatin condensation may expose the spermatozoon's nuclear DNA to damage (e.g., DNA fragmentation) during its journey through both the male and female genital tracts [40,41], and large vacuoles were found to be associated with DNA fragmentation [7,28]. However, other studies failed to establish a strong link between large vacuoles and DNA fragmentation [12,18,30,32].

This could explain the differences in terms of DNA fragmentation between different men. A disorder in spermiogenesis may result in uncondensed and vulnerable chromatin, and according to exposure to the level of oxidative stress conditions, differences in DNA fragmentation can be noticed [35].

Vacuoles and Reproductive Outcome

The importance of selecting normal spermatozoa becomes obvious when comparing the reproductive outcomes in terms of fertilization, embryo development, and pregnancy and abortion rates when oocyte injections are done with morphologically normal sperm and spermatozoa exhibiting different subcellular defects.

Selection of sperm devoid of sperm head vacuoles has multiple benefits.

Vacuoles and Embryo Development   The size and the number of sperm nuclear vacuoles, most accurately identified under Nomarski optics, negatively affect blastocyst development. In successive studies [42–45], the existence of large nuclear vacuoles, abnormal shape in spermatozoa, or both were shown to reduce the percentage of good-quality embryos reaching the blastocyst stage after culture until Day 5. Following the outcome of each embryo after injection of spermatozoa, it was clearly demonstrated that the use of spermatozoa with no vacuoles or fewer than two small vacuoles can be associated with significantly higher blastocyst rates compared with injecting spermatozoa with more than two small vacuoles or one large vacuole with or without abnormal shape. Such observations reinforce previous studies suggesting early and late paternal effects on initial embryonic development [46–48].

As mentioned, it seems that nuclear vacuoles are related to sperm DNA damage and might therefore negatively affect human embryo development [47]. In addition, it is well known that the integrity of sperm chromatin plays a key role in embryo development [25]. DNA helices in defective chromatin remodeling during spermiogenesis would be more vulnerable to physical and chemical stress such as reactive oxygen species [26]. Subsequent attack by reactive oxidative species may cause sperm's DNA fragmentation and might even affect later blastocyst development [26,49] and pregnancy outcomes in a negative manner [47,50,51].

Vacuoles and Pregnancy and Miscarriage Rates   Berkovitz et al. [52] were first to carry out a more specific analysis on the impact of sperm cells with normal nuclear shape but with large vacuoles on two matched IMSI groups of 28 patients each. Spermatozoa with strictly defined normal nuclear shape but large vacuoles were selected for injection and compared with a control group that included normal nuclear shape spermatozoa

lacking vacuoles. No difference in the fertilization and early embryo development up to Day 3 was reported. However, injection of spermatozoa with strictly normal nuclear shape but large vacuoles appeared to significantly reduce pregnancy outcomes (18% vs. 50%) and seemed to be associated with early miscarriage (80% vs. 7%).

Other studies showed that selection of normally shaped spermatozoa with a vacuole-free head was positively associated with pregnancy and lower abortion rates after Day 3 embryo transfers in couples with previous and repeated implantation failures [13,14,42,52,53] and in patients with an elevated degree of DNA-fragmented spermatozoa [23].

Knez et al. [44] showed that there was no significant difference in the pregnancy rates after IMSI and ICSI procedures and blastocyst transfer. However, after ICSI more pregnancies terminated by spontaneous abortion, whereas after IMSI there were no spontaneous abortions. One explanation could be that the IMSI procedure permits selection of spermatozoa without defects and thus provides more "healthy" blastocysts without chromosomal abnormalities, possibly in spite of very comparable development and morphology to ICSI-derived blastocysts.

Vacuoles, Health of Babies Born, and Incidence of Malformations   Still, there are concerns about the long-term safety of injecting spermatozoa carrying vacuoles. We have to be cautious, especially in the light of Aitken's work [54] on the putative negative effects of sperm DNA fragmentation for the next generation. Depending on the level of sperm nuclear DNA fragmentation, oocytes may partially repair fragmented DNA, producing blastocysts able to implant and produce live offspring. However, the incomplete repair may lead to long-term pathologies. The data of Fernández-Gonzalez et al. [55] gained on the basis of a mouse model indicate that the use of DNA-fragmented spermatozoa in ICSI can generate effects such as aberrant growth, premature aging, abnormal behavior, and tumors derived from the mesenchymal lineage that only emerge in later life.

To date, there have not been sufficient numbers of published studies concerning the health of children born after ICSI to draw any firm conclusions about the long-term safety of this procedure. However, it is important to emphasize that animal data are absolutely unequivocal on this point and clearly indicate that DNA damage in the male germ line is potentially hazardous for the embryo and therefore for the resulting offspring [56]. According to Cassuto [39], sperm nucleus morphological normalcy, assessed at high magnification, could decrease the prevalence of major fetal malformations in ICSI children.

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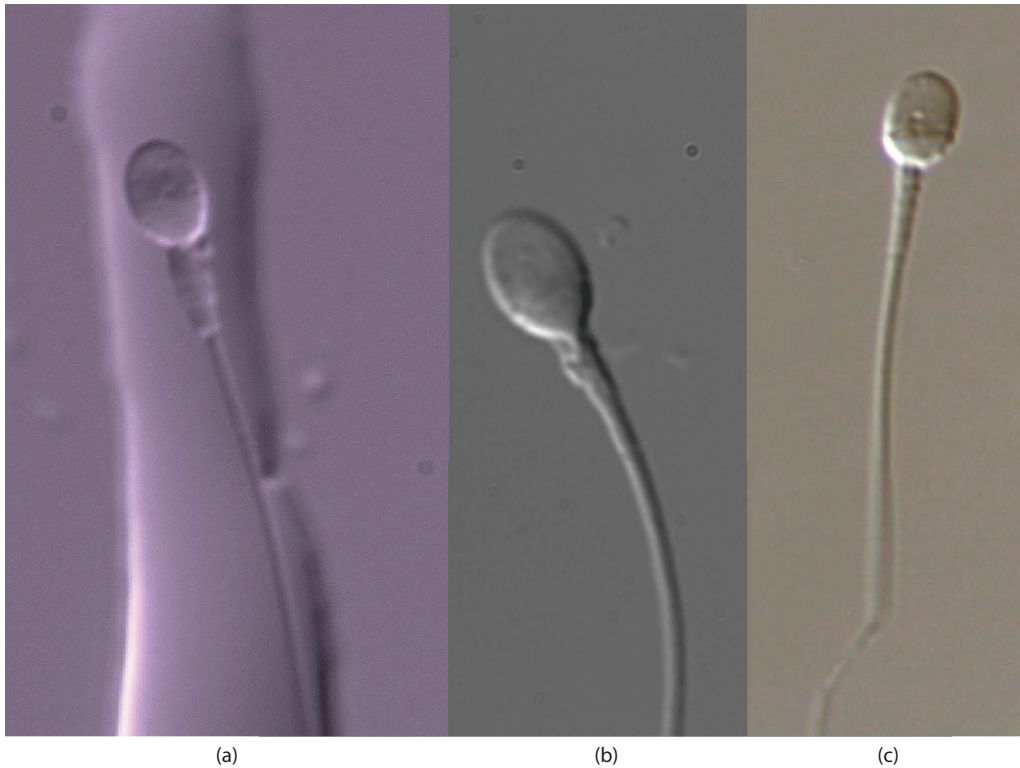
Midpiece Selection

MSOME not only permits the observation of nuclear vacuole defects but also other abnormalities that may result in infertility. Ugajin et al. [57] showed that they were able to clearly differentiate between a straight-shaped and tapering-shaped midpiece (Figure 4.2). The tapering-shaped midpiece is related to abnormal centrosomal function as a consequence of aberrant microtubule organization [57]. According to Van Blerkom and Davis [58], the centriole is, after the nucleus, the most important sperm organelle for initiation of the intraooplasmic fertilization process, being responsible for the formation of the sperm aster. The centrosomes play a crucial role in fertilization [59], and their dysfunction may cause fertilization failure due to lack of a sperm aster [60,61]. The injection of selected sperm with a morphologically straight midpiece by IMSI may result in choosing sperm with functional centrosomes, thereby positively influencing fertilization rates and embryo development after ICSI [57].

MSOME Selection in Globozoospermia to Circumvent Artificial Activation

Globozoospermia is a male-factor infertility involving several abnormalities of sperm remodeling during spermiogenesis that can lead to formation defects or premature elimination of acrosomal structures. Fertilization capacity after ICSI is low [62] mainly due to a lack of sperm-specific phospholipase C (PLCζ) [63] that is responsible for inducing Ca^{2+} oscillations essential for oocyte activation.

Presently, 21 births have been reported after ICSI followed by artificial Ca^{2+} oscillations induction via the use of assisted oocyte activation protocols by exposing oocytes to certain chemical agents (ionophore) [64–67]. Another approach proposed by Kashir et al. [68] and Gatimel et al. [67] is to apply MSOME to maximize the efficacy of ICSI and IMSI. MSOME increases the efficacy of fertilization by selecting spermatozoa with superior

FIGURE 4.2   Straight-shaped (a), intermediate-shaped (b), and tapering-shaped (c) midpieces.

oocyte activation ability. Sermondade et al. [69] selected spermatozoa that exhibited a small acrosomal bud that was not visible using conventional sperm selection methodology. Gatimel et al. [67] identified spermatozoa with some sparse oval forms, revealing the presence of Golgi residues. These studies showed that some globozoospermic spermatozoa possess an acrosomal bud that contains a total amount of PLCζ that is not significantly different from fertile controls and that correlates to successful oocyte activation and fertilization without the need for artificial oocyte activation with exogenous chemical agents.

Evaluation of Sperm Head Shape

At high magnification (6300×), Utsuno et al. [70] decomposed sperm head shapes of motile spermatozoa into four quantitative parameters: ellipticity, anteroposterior symmetry, lateral symmetry, and angularity. Stepwise forward multiple logistic regression analysis showed a statistically significant increase of the percentage of DNA fragmentation in spermatozoa with abnormal ellipticity, angularity, and large nuclear vacuoles. This may ensure the advantage of morphological assessment of spermatozoa at high magnification.

IMSI in 2013: Still a Debate

As mentioned, there is a real benefit to select morphological spermatozoa free of vacuoles. But, for different reasons, there is undeniable evident skepticism about this method of spermatozoa selection. Different doubts and interrogations may justify the hesitation to apply IMSI. What are the main indications for IMSI? How can we appreciate the benefit of IMSI?

Superiority of IMSI versus ICSI—But for Which Indications?

When should we propose IMSI instead of ICSI? In principle, all patients can benefit from using IMSI; however, it is especially useful after several previous implantation failures, in cases of severe teratozoospermia, for patients having experienced an unexplained abortion, and definitely in cases of the absence of or low blastocyst rate(s) in previous IVF attempts. However, the superiority of IMSI over ICSI is still a matter of debate. To date, randomized and well-powered studies to confirm a benefit of IMSI are limited, and they even depict conflicting results [71].

Controversial conclusions have been drawn, especially in terms of fertilization, top-quality embryo rates, and pregnancy rates between IMSI and ICSI. Some studies have shown that IMSI improves reproductive outcomes in cases of male-factor infertility and previous failed ICSI attempts in terms of implantation and clinical pregnancy rates compared with conventional ICSI [14,16,23,42,44,72,74,76,77]. Alternatively, IMSI and conventional ICSI seemed to provide comparable laboratory and clinical results when an unselected infertile population was evaluated [78,79] or when IMSI was applied as the first treatment option [80].

Full Benefit of IMSI is Attained with Blastocyst Culture

Even if the superiority of IMSI over ICSI is still a matter of debate, we have to recognize that the type of spermatozoa selected for injection influences the outcome in terms of embryo development, pregnancy, miscarriage, and malformation.

One of the main benefits of IMSI is the improvement in embryo quality and the higher rate of blastocysts obtained per cycle when morphologically good-quality spermatozoa are selected [42–45].

The type of spermatozoa selected for injection influences the outcome in terms of embryo development, pregnancy, miscarriage, and malformation.

The full benefit of a better spermatozoa selection is highlighted if we are able to produce one more blastocyst per cycle. In fact, more blastocysts provide a higher chance for the patient to achieve a pregnancy in successive vitrified embryo transfer cycle(s), thus increasing the cumulative pregnancy rate. Moreover, when using IMSI the abortion ratios are reduced by 50%, increasing as a consequence the full-term gestation possibilities.

Also, prolonged embryo culture to the blastocyst stage (5 days) can serve as a strong diagnostic tool, reflecting indications of male and female infertility and yielding useful information regarding the implantation potential of the human embryo [44].

Explaining the Discrepancies between Studies

Different conclusions may be drawn to explain the absence of differences observed by some studies. For example, for several studies, embryo transfer is performed on Day 2 or Day 3. It is fair to mention that the selection of embryos with the higher developmental potential and implantation capacity is almost impossible at this stage, even with new technologies such as the time lapse. In fact, embryo selection on Day 2 or Day 3 completely neglects the paternal effect that only becomes important after embryonic genome activation (EGA) on Day 3. It was previously shown that such "late paternal effect" negatively influences preimplantation embryo development and clinical outcomes without any detectable impairment in zygote development, such as cleaving speed or embryo quality at this stage [48,72].

Another possible explanation for these conflicting results could be the way in which conventional ICSI is performed in an individual laboratory, and how experienced the embryologists are in performing the ICSI procedure and in particular the selection of spermatozoa for injection. Collecting sperm using a 20× or 40× objective is quite common. However, at this low-magnification morphological defects of the sperm head can be hardly detected [81].

But with a well-aligned microscope equipped with 40× Hoffman contrast optics and optimal adjustment of both the optical beam and the polarizer, detection of sperm head alterations is possible. In addition, with a 1.5-fold increase in the magnification, it is practicable to observe large nuclear vacuole at 600× magnification (Figure 4.1c). However a more accurate and simple detection of small vacuoles is attained with Nomarski optics.

From our own experience, we realized after some months of application of MSOME-IMSI that it was possible to observe nuclear defect even with a classical objective. When we started to analyze in a sibling study the rate of blastocysts after IMSI or ICSI, we observed a significant difference of almost 25% in favor of IMSI. With the increasing experience and being aware of the presence of a vacuole, the probability to select, with the conventional ICSI microscope, spermatozoa free of a vacuole was increased. This has as a consequence an increase in the rate of blastocysts after ICSI and attenuation in the difference between both techniques.

The conclusions of such observations are that regardless of potential indications of IMSI, we have to select the best spermatozoa possible and exclude those carrying nuclear defects for all male infertility patients. Of course, not all spermatozoa with a nuclear vacuole will have a negative impact on the developmental rate, but if morphologically normal spermatozoa are present in the suspension, it is mandatory to try to select them independently of the technique that is available.

Thus, we have to do all we can to select the best spermatozoa. There are absolutely no indications to select bad-quality spermatozoa if good spermatozoa are present in the prepared semen sample. Are there still indications where improved sperm selection before fertilization is not necessary or low-magnification microscopy using HMC is more than enough? Most probably this is not the case.

Technical Aspects

Several reservations concerning technical aspects serve as arguments to not apply this method of spermatozoa selection:

1. Too sophisticated system? Too complicated to perform.
2. Lack of standardized sperm selection criteria according to MSOME (classification).
3. Time-consuming aspect during spermatozoa selection.
4. Prolonged selection time of male gametes at the expense of oocyte aging
   a. Problems in managing assisted reproductive technology (ART) laboratory
   b. High cost

The aim of our work is first to use this technology in a user-friendly way and second to classify the semen and provide in real time a spermocytogram (MSOME), and for the IMSI technique to select spermatozoa in a manner that does not impair oocyte quality.

Materials

Basic Materials

Observation at high magnification (MSOME) or sperm selection (before oocyte injection) is performed on an inverted light microscope equipped with DIC/Nomarski DIC optics, with dry or immersion 63× or 100× magnification objectives lens. In our IVF units, we use a Leica 6000 (Leica Microsystems, Germany) equipped with 63× and 100× DIC objectives.

Dry objectives instead of immersion are more convenient when dishes or slides are moved or replaced for each oocyte injection. Fast motion or dynamic movements between drops induce air bubble formation between the objective lens and the dish, preventing the visualization of the semen sample. The use of dry objective permits an easy handling of the dishes, in particular when a new dish is used for each oocyte injection.

Additional Materials

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Using a variable zoom lens (HC Vario C-mount; Leica Microsystems) and subsequent magnification of the image with a high-definition digital video camera makes it possible to evaluate spermatozoan morphology on a monitor at magnifications between 6600× and 12,000× in real time or after spermatozoa immobilization.

Despite all the available equipment and computer software, there is not a high priority to perform such observation and selection of spermatozoa using high magnification (>6000×) in conjunction with digital image capture systems to analyze spermatozoa in detail after their capture and visualization on a color monitor screen.

Practical and technical aspects of sperm selection need to be considered to facilitate the workflow while performing IMSI.

For IMSI, inverted microscopes are equipped with the classical ICSI equipment (Narashige or Eppendorf micromanipulators and injectors). Calculation of the total magnification on the screen monitor depends on several technical specifications of the objective, the magnification selector of the microscope, the variable zoom lens, the camera chip diagonal dimension, and the television monitor diagonal dimension.

MSOME: A Useful Tool for Routine Laboratory Semen Analysis

### Morphological Normalcy of Spermatozoa Assessed by MSOME

Based on electron microscopy, and high-magnification optic microscopy, Bartoov et al. [10] defined the morphological normalcy of motile-sperm nucleus according to the shape and chromatin content. The shape has to be smooth, symmetric, and oval, with average length and width limits estimated to 4.75 ± 0.28 µm and 3.28 ± 0.20 µm, respectively (Figure 4.3).

The chromatin mass has to be homogeneous and should contain no extrusions or invaginations, with a maximum of one vacuole involving <4% of the nuclear area. The acrosome and postacrosomal lamina are considered abnormal if absent, partial, or vesiculated. An abaxial neck with the presence of disorders or cytoplasmic droplets as well as the presence of broken, short, or double and coiled tail is considered abnormal.

Establishment of an MSOME Sperm Classification

According to Bartoov et al. [10], normal spermatozoa should not contain nuclear vacuole(s) exceeding >4% of the nuclear area. Saidi et al. [82] and Perdrix et al. [83] classified the relative area of the vacuole into
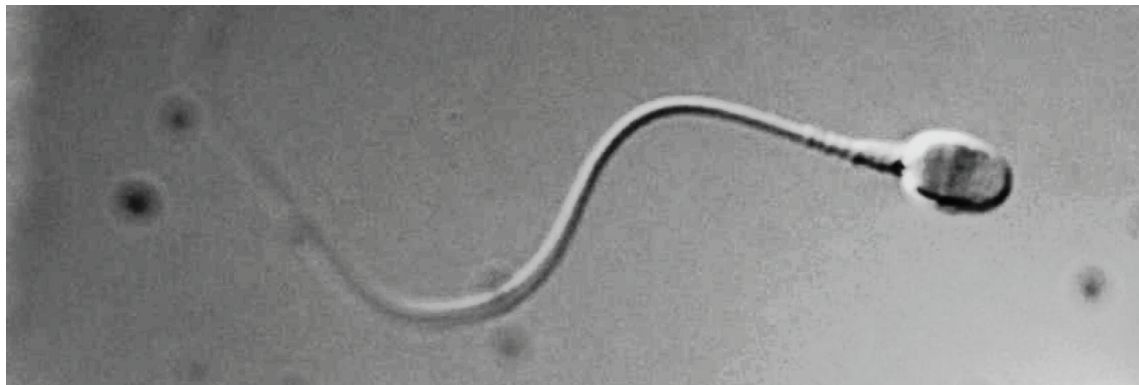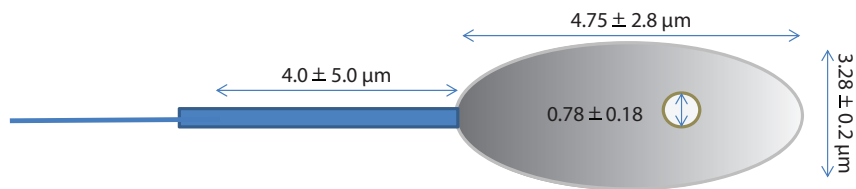

FIGURE 4.3 Representation of a normal spermatozoon according to Bartoov criteria.

three groups: (1) <5.9%–6.5%; (2) between 6.5% and 13% or between 5.9% and 12.4%; and (3) >13% or 12.4%, respectively. For Franco et al. [28], large nuclear vacuoles in spermatozoa were defined by the presence of vacuoles occupying ≥50% of the sperm nuclear area.

Under those circumstances where no normal spermatozoa in the semen sample can be found, the only alternative is then to select the morphologically "second-best spermatozoa." Hence, it is essential to know from the second-choice spermatozoa with vacuoles, abnormal shape, or both as to which spermatozoa we have to select. It seemed that the establishment of a classification system of the spermatozoa according to different types of abnormalities was mandatory for the selection of spermatozoa in a more tangible way and also to analyze their influence on further outcomes.

To estimate the impact of specific sperm defects on embryo development and further outcomes in an accurate way, different groups established models of sperm classification according to the normalcy of the shape and the presence and size of vacuoles [42,43]. For example, Vanderzwalmen et al. [42] classified spermatozoa into three grades according to the presence and size of vacuoles: Grade I, normal shape and a maximum of two small vacuoles; Grade II, normal shape and more than two small vacuoles or at least one large vacuole; Grade III, abnormal head shapes with or without large vacuoles in conjunction with other abnormalities at the level of the base (Figures 4.4 and 4.5) [42].
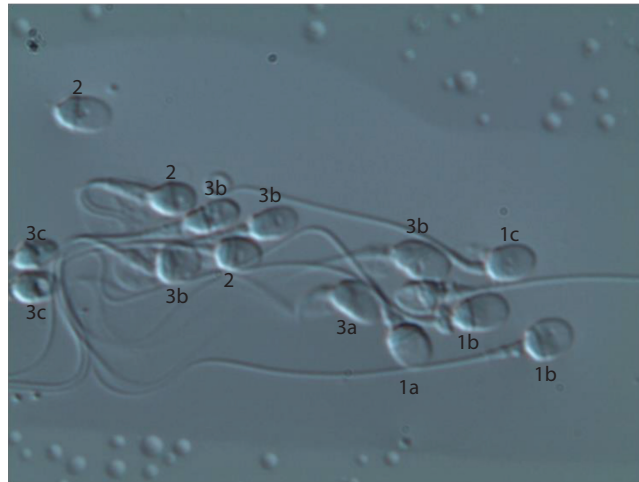
FIGURE 4.4 Spermatozoa vacuolization classification according to Vanderzwalmen et al. [42], observed with Nomarski optics 63× dry objective. Class I spermatozoa with normal head shape without vacuole (1a), with one vacuole (1b), and with maximum of two small vacuoles (1c). Class II spermatozoa with large nuclear vacuole (LNV) (2). Class III spermatozoa with abnormal shape without vacuole (3a), small vacuoles (3b), and LNV (3c).

(a)   (b)
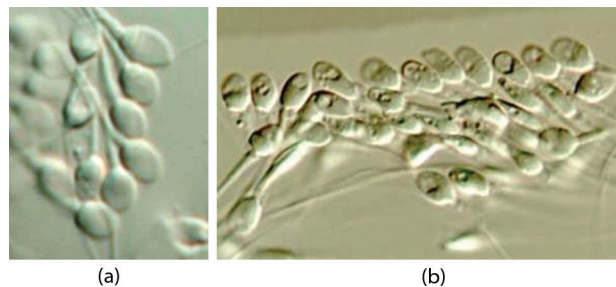
FIGURE 4.5 Spermatozoa vacuolization pattern. (a) High frequency of class I spermatozoa (fertilization after conventional insemination). (b) Severe teratozoospermia (100 spermatozoa class III).

Cassuto et al. [43] established a detailed classification scoring scale ranging between 6 and 0 points according to the normalcy of the head (2 points if normal), the symmetry of the base (1 point if normal), and the absence of vacuole (3 points if absent). Three classes of scoring were therefore established: class 1, high-quality spermatozoa with calculated score of 4–6; class 2, medium-quality spermatozoa with calculated score of 1–3; and class 3, low-quality spermatozoa with calculated score of 0.

MSOME for Routine Laboratory Semen Analysis

To assess the usefulness of the evaluation of sperm morphology by MSOME, two studies were undertaken in a first instance by Bartoov et al. [10,13] that estimated the correlation between MSOME and the World Health Organization (WHO) routine method. More recently, Oliveira et al. [84] compared the MSOME evaluation with the Tygerberg classification criteria.

Both works conclude that the MSOME criteria appear to be much more restrictive, presenting significantly lower sperm normalcy percentages for the semen samples compared with those found after routine analysis by WHO criteria and the Tygerberg classification. In addition, MSOME represented a much stricter evaluation, because the use of Nomarski optics enabled the identification of vacuoles that could not be described with the same accuracy with other methods.

These studies point toward a benefit in sperm morphology and quality evaluation by including MSOME among the criteria for routine laboratory semen analysis before ICSI or conventional IVF procedures. A previous MSOME spermocytogram revealing a high percentage of vacuoles may be judicious to propose directly IMSI as the best therapy for ICSI candidates. Furthermore the additional information gained by MSOME may help to avoid fertilization failure in IVF cycles.

Proposed Cutoff Threshold for Further Treatment Decisions

With the aim to define a predictive value of sperm normalcy using MSOME on the outcome of combined IVF-ICSI, Wittemer et al. [85] undertook a study including 55 couples with previous failure of implantation after intrauterine insemination (IUI) treatments. In their next attempt, a combination of conventional IVF and ICSI was proposed for each couple. They concluded that below a threshold of 8% of morphological normal spermatozoa observed by MSOME, ICSI must be performed instead of conventional IVF to avoid the risk of fertilization failure. Cassuto (personal communication) observed that if <42% of score 6 spermatozoa (equivalent to our class I classification) are present in the semen sample, ICSI-IMSI has to be performed instead of conventional IVF.

Falagario et al. [86] identified a cutoff of 20, or 28% of sperm nuclear vacuolization (SNV) on the total of sperm in a seminal sample as a physiological threshold. In their study, they observed that patients undergoing ICSI, grouped according to SNV, showed similar percentages of fertilization and embryo development after Day 2 embryo transfer but that more pregnancies were achieved with a higher implantation rate when SNV was lower or equal to 20 (28%). They concluded that SNV rate could be introduced as an easy diagnostic evaluation before performing an ICSI–IMSI cycle.

Sperm Preparation for Morphological Observation

For MSOME, observation of the spermatozoa may be done on the native semen or after the sperm washing procedure on a three-layer gradient of pure sperm (Nidacon, Sweden), as described previously [87]. It should be stressed that MSOME was applied exclusively to motile spermatozoa that under low-light microscopy magnification have a high potential to be selected for ICSI.

How to Perform MSOME

In certain situations, when we have to decide quickly after the oocyte pick-up whether IVF or ICSI-IMSI has to be performed, a rapid assessment of the morphology may be a good decision. If MSOME was part of a previous

exam (e.g., spermocytogram), assessment of the morphology is done in a more sophisticated way with the microchannel capture technique.

Rapid Morphological Assessment

To receive a rapid qualitative evaluation of the semen sample, a fast morphological assessment is performed using a microscope coverglass (24 × 60 mm). Elongated drops (~5–10 µL) of 3%–10% polyvinyl pyrrolidone (PVP) solutions are placed on the coverglass (Figure 4.6a). An aliquot of (1–10 µL) of native or washed semen sample is transferred on the border of one elongated drop (Figure 4.6b). In case of too low sperm concentration or too low motility, drops of semen sample are placed in the three elongated drops of 3% PVP.

When spermatozoa start to swim, the evaluation of the morphology of motile spermatozoa is done in real time. According to the initial concentration, 50–100 spermatozoa are observed, classified, and recorded in a file (Figure 4.7).

Morphological Examination Using the Microdrops Capture Channel

In cases where quantitative evaluation of the semen sample is needed for research purposes, elongated drops of culture medium are placed into a glass-bottomed dish (GWST 5040; WillCo, World Precision Instruments Wells BV, the Netherlands) and covered with mineral oil (Irvine Scientific, Ireland). The number of elongated drops depends on the semen quality. With reduced sperm quality (severe oligoasthenozoospermia), more elongated drops are prepared.

After the washing step, the sperm suspension is deposited on the border at one end of the elongated drop. When motile spermatozoa start to swim along the edge of the elongated drop, sperm capture channels are created

Microscope
cover glass

Elongated PVP drops

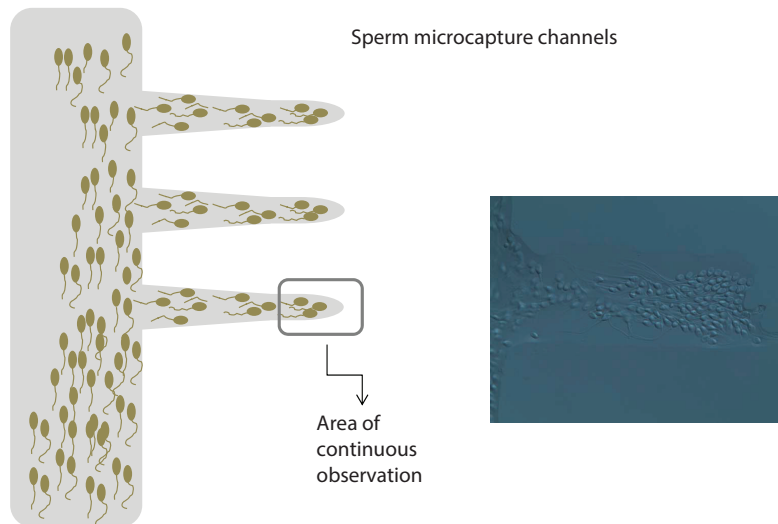(a)   (b)
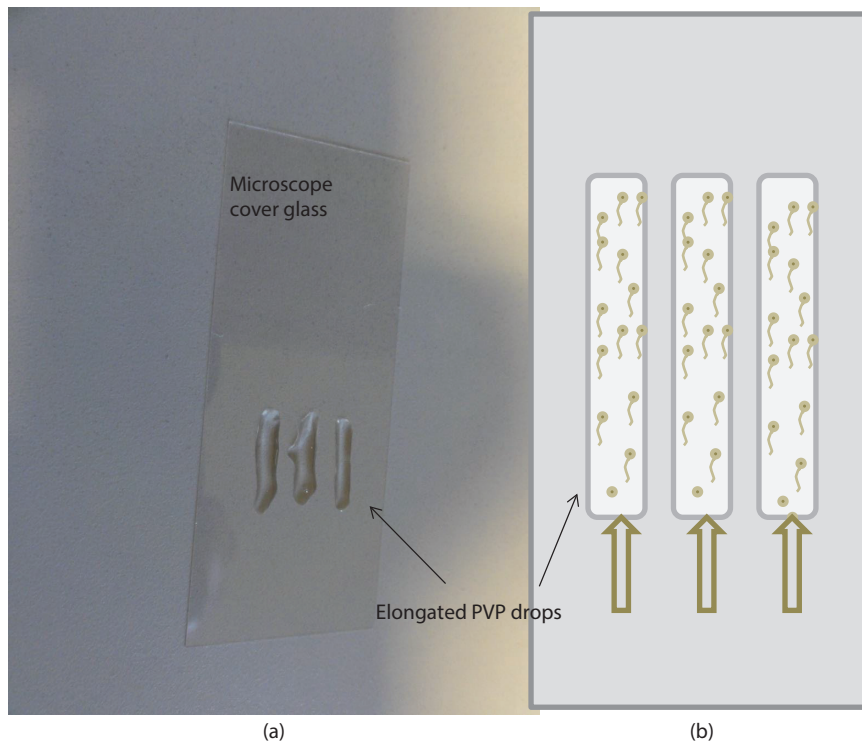
FIGURE 4.6   (a) Preparation of the slide with PVP drops for MSOME. (b) Arrow indicates where the microdrop of semen is deposited.
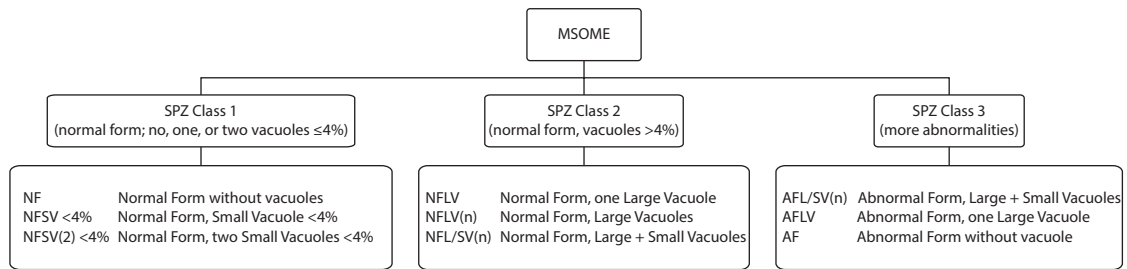
FIGURE 4.7 Flow chart reporting the percentages of spermatozoa according to the different vacuolization patterns.

FIGURE 4.8 Application of the sperm-microcapture channels, where spermatozoa accumulate for MSOME.

by pulling channels from the drop with a microinjection pipette ICSI pipette (Microtech, Czech Republic) that is fixed on the micromanipulator [88] (Figure 4.8).

A proper setup of the dish for selecting and capturing sperm facilitates the routine application of IMSI.

Motile spermatozoa enter the small bays and accumulate at the end, where they stop their progressive movement due to a restriction of the free moving space.

A total of eight channels per elongated drop are created and examined (Figure 4.8). Because the majority of the spermatozoa present at the end of the channel show no progressive motility, it is possible to follow them and take several pictures. Morphological assessment of the spermatozoa may be performed in real time under the microscope at magnifications between 630× and 1000×.

A subsequent analysis is conducted on the monitor screen after taping the channel using time-lapse recording (Leica Application Suite magnification optics; Leica). Nevertheless, static sperm imaging only allows evaluation of the visible part of sperm and might leave some morphological alterations undetected.

A minimum of 100 spermatozoa were analyzed and documented on a specific file (Figure 4.7).

Assessment with a Transparent Celluloid Form

Bartoov et al. [13] advised assessing the normalcy of spermatozoa by superimposing a fixed transparent celluloid form on the motile examined gametes. This fixed, transparent, celluloid form represents a sperm nucleus fitting the criteria of normalcy of the head (average length, 4.75 ± 0.28 μm; average width, 3.28 ± 0.20 μm). The correct sperm size, calculated by the ratio of expected normal sperm size to the actual size, can be visualized on the monitor screen. The nuclear shape is documented as abnormal if it varies in length or width by 2 SDs from the normal mean axes values [13]. This way of analyzing the morphology is extremely precise. The disadvantage of this technique is that it is very time-consuming and difficult in practice to compare the spermatozoa fitting the criteria of normalcy with moving spermatozoa.

IMSI

Sperm Preparation before IMSI

Before IMSI, only washed semen was used after density-gradient centrifugation on one to three layers of pure sperm (Nidacon, Sweden) [87]. The number of layers depended on the initial sperm concentration.

Two Strategies to Perform IMSI

Three criticisms about IMSI are frequently argued. The first concerns the prolonged period of time for oocytes out of the incubator during spermatozoa selection. The second criticism is that the prolonged selection time of male gametes may be at the detriment of oocyte and promote oocyte aging. Finally, some argue that exposing the spermatozoa for a longer period at 37°C increases the rate of vacuoles. To reduce the time of the oocytes out of the incubator, two approaches were implemented, as presented below.

First Approach: Sperm Selection and Oocyte Injection on Two Different Microscopes

The spermatozoa are selected using the Nomarski optic, and oocyte injection is performed with a conventional ICSI microscope. With such a strategy, the oocytes are not present in the dish during selection of the spermatozoa. After selection, the dish is incubated for 15–30 min to stabilize the temperature, pH, or both.

In cases of severe teratozoospermia, taking into account that more time for sperm selection is needed, we plan the selection at least around the time of oocyte pick-up (OPU). With such a policy, oocyte injection can be performed 2–3 hr after OPU (38–40 hr post-human chorionic gonadotrophin [hCG] administration) on the ICSI station, thereby avoiding oocyte aging. Another advantage of this approach concerns the organization of the laboratory work. In cases of several IMSI, the IMSI station is only occupied for the selection process and not for the injection phase. Also with this approach, the IMSI microscope is not occupied for an excessive period, and the oocytes are removed from the incubator only for the injection step.

Another possibility is to select the spermatozoa in a glass-bottomed dish and place them directly in a conventional ICSI dish.

Preparation of IMSI Dishes   Under sterile conditions, several drops are deposited in the glass-bottomed dishes (Figure 4.9).

Sperm selection PVP drops (A)
On the left side of the dish, place one to three elongated drops of 7.5%–10% PVP (~5–10 μL). In this drop, selection of the spermatozoa is performed. The number of drops depends on the quality of the semen that was assessed in a previous MSOME analysis or on the number of metaphase II (MII) oocytes, which are needed for injection. The aim is that the spermatozoa that we select stay in the PVP drop for a period that does not exceed 15 min. Moreover, according to this rule, several elongated drops and even IMSI dishes should be prepared if many MII oocytes are present.

Sperm aliquots drops (B)
Under each sperm selection drop (A), a drop of ~10 μL of human tubal fluid (HTF)-HEPES (IVFonline, Canada) containing 6% human synthetic albumin (HSA) (Irvine Scientific) is deposited.
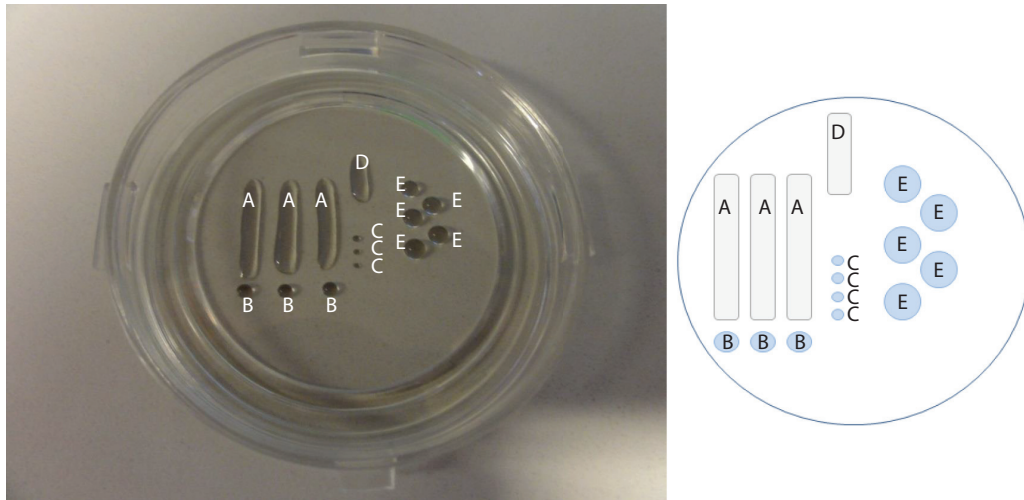
FIGURE 4.9 Position of the drops in IMSI dishes (A, D) 7.5%–10% PVP. (B, C, E) Culture medium. (A) Sperm selection PVP drops. (B) Sperm aliquot drops. (C) Host-selected spermatozoa microdrops. (D) Sperm immobilization drops. (E) Oocytes injection drops.

Host-selected spermatozoa microdrops (C)
Adjacent to the elongated drops of PVP (A), very small drops (<1 μL) (C) of HTF-HEPES containing 6% HSA are deposited with a small stripper pipette. The microdrops will host the spermatozoa that were selected in drop A until oocyte injection.

Sperm immobilization drops (D)
A small drop of 10% PVP in which sperm immobilization will take place is deposited in the upper part of the dish.

Oocytes injection drops (E)
The right side of the dish contains five drops of HTF-HEPES containing 6% HSA in which oocyte injection will take place following the policy of IMSI procedure.

All drops were covered with sterile mineral oil (Irvine Scientific).

Method of Spermatozoa Selection and Injection   The entire selection process is performed at room temperature. At the beginning of IMSI, a small aliquot of washed sperm (1–10 μL according to the semen sample quality) is transferred into drop B (Figure 4.10a) and a small bridge is formed between drop B and drop A (Figure 4.10b). The motile spermatozoa swim into the PVP drop and the morphologically normal spermatozoa (or the best second class) are selected with an ICSI pipette and transferred into a small drop of culture media (drop C) (Figure 4.10c). They are selected at a magnification of 1000×.

After collecting spermatozoa (if possible, 1.5 times the number of oocytes to inject), the dish is removed from the IMSI station and placed on a 37°C heating stage for temperature recovery (~30 min).

After this period of incubation at 37°C, the oocytes are placed into the culture media (E), and ICSI is performed using a conventional Hoffman microscope at 400× magnification (Figure 4.10d). Motile spermatozoa are aspirated from the preselected host drop (C) and immobilized in the PVP drop (D) before injection.

Second Approach: Sperm Selection and Oocyte Injection on the IMSI Station

When the purpose of the study is to follow the outcome of embryo development in relation to the type of injected spermatozoon (photodocumentation of the injected spermatozoon), a maximum of two oocytes are placed directly in the IMSI dish (Figure 4.11). A minimum of two dishes are prepared as presented previously, except that sperm host drops are not present before starting such an approach, the quality of
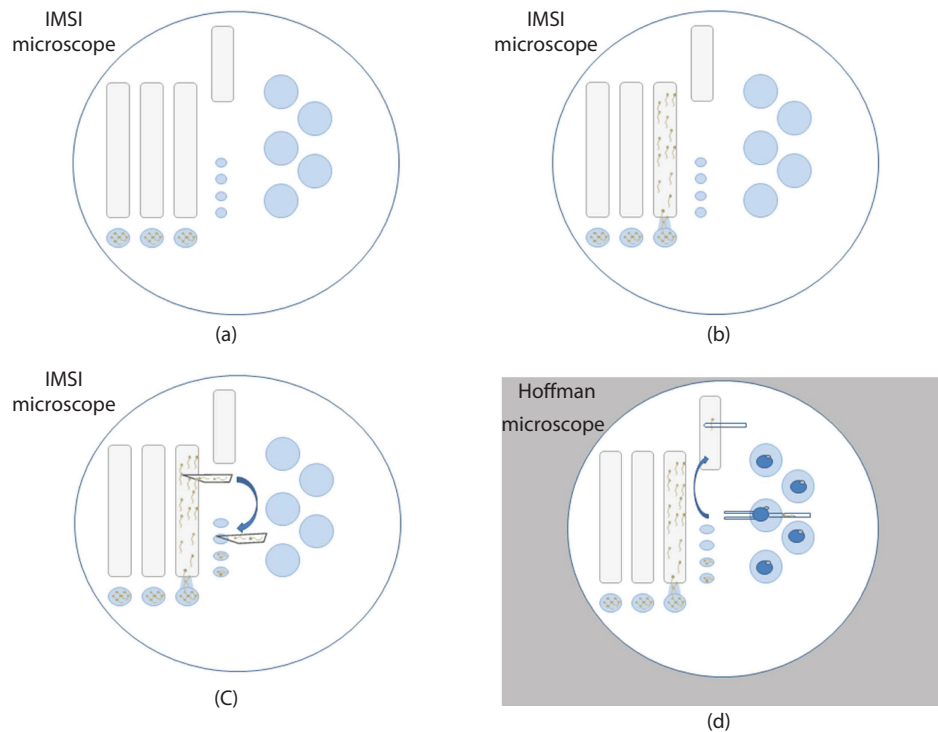
FIGURE 4.10 IMSI. Description of the different steps for sperm selection on the IMSI microscope (a–c) followed by oocyte injection on the Hoffman ICSI microscope (d).

the spermatozoa should be assessed to be sure that the time spent to select one class I or II spermatozoa will not exceed 2 min.

This procedure is performed at 37°C. At the beginning of the IMSI procedure, a small aliquot of washed sperm (1–10 μL) is transferred into drop B and a small bridge is formed between drop B and the elongated selection drop. The motile spermatozoa swim in the PVP drop, and the motile spermatozoa are morphologically selected at a magnification of 1000× (Figure 4.11a) before immobilization in drop D (Figure 4.11b). Use of a variable zoom lens (HC Vario C-mount; Leica) allows for the reevaluation (after immobilization) of the morphology on the monitor at magnifications between 6600× and 12,000× and the performance of photodocumentation for a subsequent analysis. Injections are performed directly after immobilization in the same dish. After IMSI, single drop culture (in a petri dish or with the time-lapse technology) is performed.

The next two oocytes are placed in a new IMSI dish prewarmed on the heating stage. The previous IMSI dish is then placed on a heating stage at 37°C until the next injection selection and injection step. This technique is easy to apply with dry objectives, so that no problem with oil occurs when changing dishes.

Time Spent to Select Spermatozoa

Regarding time allotted to select spermatozoa, the primary intention is to choose normal-shaped spermatozoa without any vacuoles (class I) for injection into the oocytes. Depending on the degree of impaired sperm morphology, the mean time required selecting the best sperm ranged between 2 and 15 min. However, when it is obvious after 15 min of sperm examination that spermatozoa of a normal morphology cannot be found, the second-best spermatozoa with the least number of vacuoles, other abnormalities, or a combination is selected for injection.
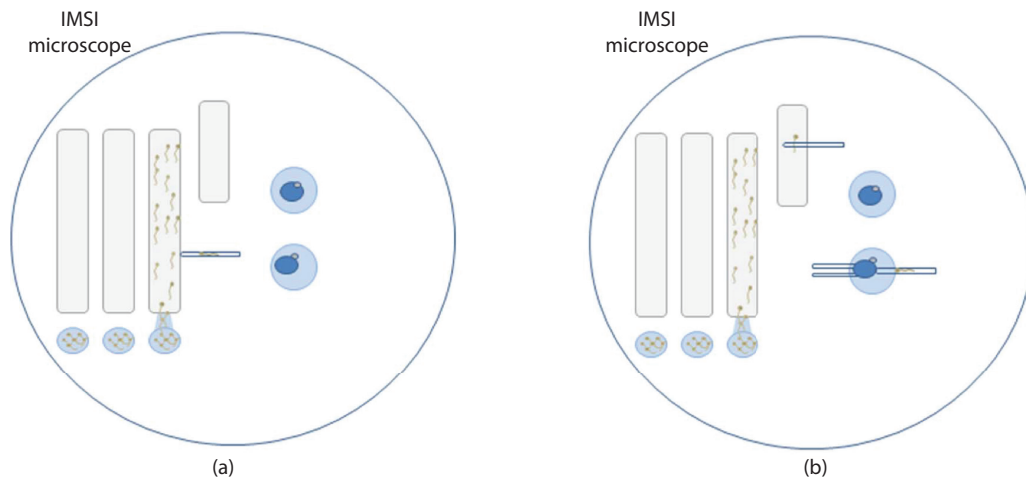
FIGURE 4.11 IMSI. Description of the different steps for sperm selection (a) and oocyte injection on the IMSI microscope (b).

In such situations, it might be difficult to decide whether to stop the search for a normal spermatozoon or to proceed. It might take 15 min and even longer; of course, this also depends on the number of oocytes that have to be injected and on the number of patients that have to be treated.

Temperature for Sperm Evaluation or Selection

Another crucial question is whether the duration of sperm selection and the temperature might influence vacuole formation. A study by Peer et al. [89] demonstrated that after 2 hr of incubation at 37°C in culture media, the incidence of spermatozoa with vacuolated nuclei was significantly higher compared with incubation at 21°C. Peer and colleagues suggested that prolonged (≥2 hr) sperm manipulations for ART should be performed at 21°C rather than 37°C. Schwarz et al. [90] reported a significant increase in SNV in washed sperm but not in swim-up sperm. They concluded that the mode of sperm preparation does influence SNV and that vacuolization is unaffected by temperature in motile sperm isolated by swim-up.

Several experiments were conducted in our centers to analyze the formation of vacuoles in real time. We implemented a time-lapse recording approach on selected Grade I spermatozoa incubated for 24 hr at 37°C. Compared with the control group, no changes in the morphology of the spermatozoa were observed. No vacuoles appeared. Even when the same experiment was conducted on spermatozoa with vacuoles, no changes in the size and shape of the spermatozoa after 24 hr incubation at 37°C were found [88].

Conclusions

One of the most essential questions is not under which technical conditions the selection of spermatozoa should be recommended but rather whether we have to consider to select the best spermatozoa and, if possible, exclude those carrying defects.

For this reason, MSOME and IMSI were developed. MSOME appears to be a powerful method to improve our understanding of human spermatozoa, and IMSI is now routinely used in ART practices.

However, the clinical use of MSOME and IMSI remains unclear in the fields of male infertility diagnosis and ARTs. Answers to a lot of questions are pending or unclear for the following issues: (1) the terminology of vacuoles, their classification, and their location on the sperm head; (2) vacuole origin and formation; (3) application of MSOME-IMSI for specific indications, such as teratozoospermia or to a large population; (4) the application of IMSI instead of IVF in cases of unexplained infertility; and (5) oocyte repairing

factors. Nevertheless, it is increasingly obvious that large vacuoles reflect a pathological situation, most probably correlated with sperm chromatin immaturity. In addition, we have to be aware that this technique is challenging and has to be performed under the best working conditions so as to not impair the quality of oocytes.

The introduction of IMSI made embryologists aware that in times of ICSI the selection of sperm has to be given proper attention.

The introduction of IMSI has the advantage that embryologists realize that more attention has given to sperm selection even in cases of classical ICSI. The application of IMSI leads to more and better quality blastocysts and thus it increases the chance to select the proper embryo for transfer with the highest implantation potential. The full benefit of using MSOME as an additional tool to ICSI procedure manifests when it is performed in combination with Day 5 embryo culture of all fertilized oocytes. Also, knowing the excellent results obtained with vitrification should not be underestimated.

Even though there are only few reports in the human species on the abnormal outcome generated by spermatozoa carrying vacuoles, a higher and better-resolution technique has to be added as an additional tool for ICSI, knowing the possible consequence of sperm DNA damage for the offspring. Presently, one study reports higher *de novo* malformation after ICSI selection than after IMSI selection [39].

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