—Mini Review—

Sperm selection method in ICSI

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Abstract: Since the establishment of intracytoplasmic sperm injection (ICSI) in 1992, sperms have been observed, evaluated, and selected under an inverted microscope at 400 × magnification for ICSI. Intracytoplasmic morphologically selected sperm injection (IMSI) is a technique in which sperms are selected for micro insemination by more precisely observing their morphology, at increased magnification under an inverted microscope used for micro-insemination and enhancing the image resolution. Approximately 20 years have passed since IMSI was developed, and its efficacy has only been confirmed in cases with a high rate of sperm aneuploidy, a high rate of sperm DNA fragmentation, and repeated ICSI failures. It is well-known that the quality of sperms has a profound effect on the quality of embryos which may eventually become children. Therefore, when micro-insemination is performed, sperms should be selected by not only paying attention to their motility, but also by closely observing their morphology. Those engaged in fertility treatment should always keep in mind that sperm selection itself has a significant influence on patients' lives.

Key words: micro insemination, ICSI, IMSI, Sperm selection, High power microscope

Introduction

In recent years, more and more women have given birth after fertility treatment using assisted reproductive technology (ART), and various techniques have been developed, such as intracytoplasmic sperm injection (ICSI), blastocyst transfer, embryo vitrification, time-lapse embryo observation, and new culture media. With the development of these techniques for assisted reproductive technology, clinical performance has dramatically improved. However, the most important factor in achieving pregnancy is whether an embryo with the potential to develop into a child can be obtained, and the quality of such embryos is largely influenced by the qualities of the ovum and sperm.

Methods of obtaining more ova in good condition have been developed. Ovulation-inducing drugs and gonadotropin-releasing hormone agonist (GnRHa) have been introduced to clinical practice, and improvements to embryo culture systems have made it possible to obtain good blastocysts at a high yield. In association with this, single embryo transfer (SET) has become the principal treatment in ART

Regarding assisted fertilization technology related to sperm, ICSI using human sperms was a remarkable innovation which was first successfully performed by Palermo *et al.* in 1992. When ICSI was first developed, sperms were selected mainly based on their motility. In recent years, it has become possible to easily culture embryos to the blastocyst stage, and it has been frequently reported that sperms are involved not only in fertilization, but that the quality of sperm affects the development of embryos up to the eight-cell stage, and the genetic components of sperm including chromosomes greatly affect the formation of the blastocyst after the morula stage. Furthermore, considering that a high incidence of pediatric cancer has been reported among children conceived with sperm of male smokers, it is indisputable that the quality of sperm affects the quality of embryos, pregnancy, and also the growth and development of the child.

Although the size of a sperm is 20 times smaller than that of an embryo or an ovum, sperms used in current ICSI are observed, evaluated, and selected under an inverted microscope at the 400 × magnification usually used for observing embryos or ova. Intracytoplasmic morphologically selected sperm injection (IMSI) is a technique in which good sperms are selected for micro insemination by more precisely observing their morphology (particularly the presence or absence of sperm head vacuoles) at increased magnification under an inverted microscope used for ICSI and enhancing the image resolution.

This paper will reviews actual practice of IMSI performed at our clinic and the literature of IMSI results.

Received: January 20, 2015
Accepted: March 3, 2015
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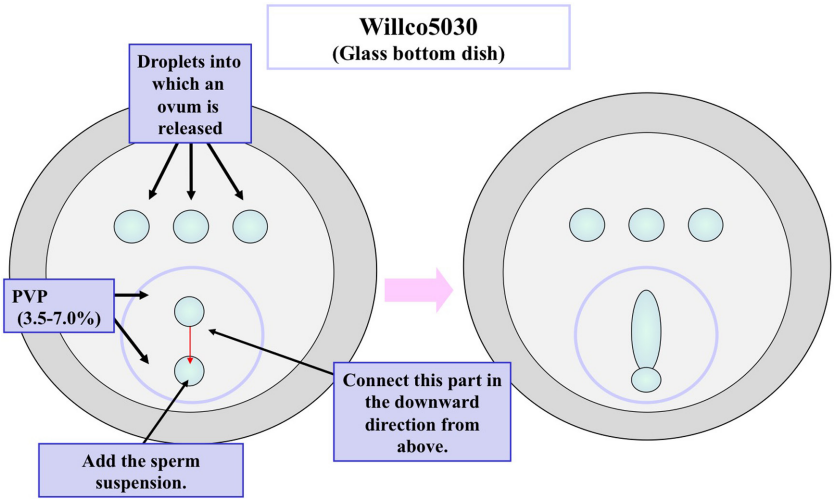

Fig. 1. Preparation of the IMSI dish.

The Practical Apsects of IMSI

Preparation of the IMSI dish

Because sperm morphology is observed by the differential interference contrast (DIC) method using a 100 × oil-immersion objective lens, it is necessary to make a hole on the microscope hot plate and select a glass bottom dish for the IMSI dish. At our clinic, a Willco5030 is used as the glass bottom dish. Due to the very small amounts of the droplets to be prepared, each droplet is prepared after filling the dish with oil (OVOIL, Vitrolife). A circle is drawn one size smaller than the hole on the microscope stage on the back side of the dish, and within this circle, two droplets of 3.5–7% polyvinylpyrrolidone (PVP) are deposited for the sperm suspension. Outside the circle, three droplets of G-MOPS (Vitrolife) are deposited for the ova. The dish is placed in a 37 °C incubator and kept it warm until the start of IMSI. If there are six or more metaphase II (MII) ova, at least two dishes are prepared and used alternately in order to prevent a temperature drop. When a dish is not being used, it should be returned to the incubator to maintain the temperature at 37 °C. Ten mins before starting IMSI, the undiluted sperm suspension prepared in advance is added to the lower one of the PVP droplets created. The amount of sperm suspension to be added is adjusted taking into account the final concentration of the undiluted sperm suspension as well as the sperm motility rate. After the undiluted sperm suspension has been added, the two droplets are connected by creating a bridge from the other PVP droplet to the PVP droplet to which the undiluted sperm suspension has been added (Fig. 1). The dish is returned to the 37 °C incubator to maintain the temperature, and to wait for the sperms with good motility to swim up to the connected droplet (Fig. 2).

Evaluation of sperm morphology

Sperm morphology is evaluated using a unique grading system at our clinic. Based on the data reported by Bartoov *et al.*, the size of vacuoles is determined by whether they are larger or smaller than 4% of the sperm head surface area. The size of 4% is confirmed by drawing 25 circles on the sperm head image (Fig. 3). In addition, the number of vacuoles is recorded after the evaluation. The sperms are classified according to the following five grades, A to E.

Evaluation A includes sperms that have a normal head, a midpiece, and tail morphologies without vacuoles;

Evaluation B includes sperms that have a normal head, a midpiece, and tail morphologies with vacuoles smaller than 4%;

Evaluation C includes sperms that have a normal head, a midpiece, and tail morphologies with vacuoles 4% or larger; and

Evaluation D includes sperms that have a normal head, a midpiece, and tail morphologies with both large and small vacuoles.

Evaluation E is defined as sperms other than the above (Fig. 4).

IMSI procedure

First, the sperms for IMSI are briefly observed in order to determine in advance if good quality sperms can easily be selected (spermocytogram using the simplified meth-

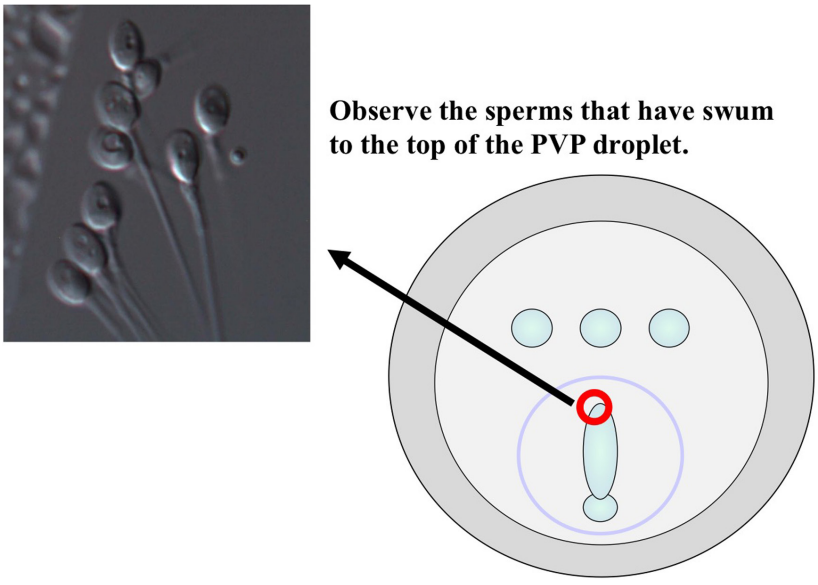

Fig. 2. Sperms that have swum to the top of the PVP droplet.

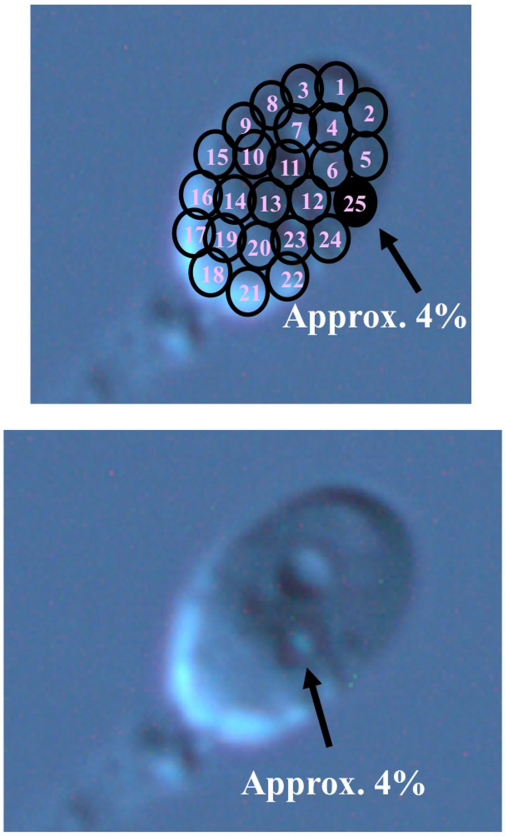

Fig. 3. Confirmation of a vacuole of approx. 4%.

od). Immersion oil is applied to the back of the IMSI dish which is then positioned on the microscope stage where the hole has been made. The sperm morphology is observed using a 100 × oil-immersion objective lens. If it is determined to be possible to easily select sperms with a sperm morphology of A or B (i.e., possible to select them in approximately five mins), ova are added to the ovum droplets on the IMSI dish. If it is difficult to select A or B sperms, ova are added to the IMSI dish after sperm selection. This paper describes the IMSI procedure when it is determined to be possible to select A or B sperms easily.

The ova are placed in the ovum droplets in the IMSI dish and immersion oil is applied to the back of the dish. The sperm suspension droplets are aligned in the IMSI dish with the center of the hole on the microscope hot plate. Then, the IMSI dish is positioned so that the ovum droplets are located outside the hole, or in other words on the hot plate, to prevent the temperature of the ovum droplets from dropping (Fig. 5). In order to accelerate the movement between the sperm suspension droplets and ovum droplets under high magnification, the ovum position and the observation position of the sperms (the top of the sperm suspension droplet) on the Leica microscope stage should be stored in the PC coordinate storage system, because the visual field narrows as the magnification increases, and storing the coordinates enables smoother and more rapid positioning of the ova after the sperm has been extracted. The microscope field is moved to the stored position or the top of the sperm

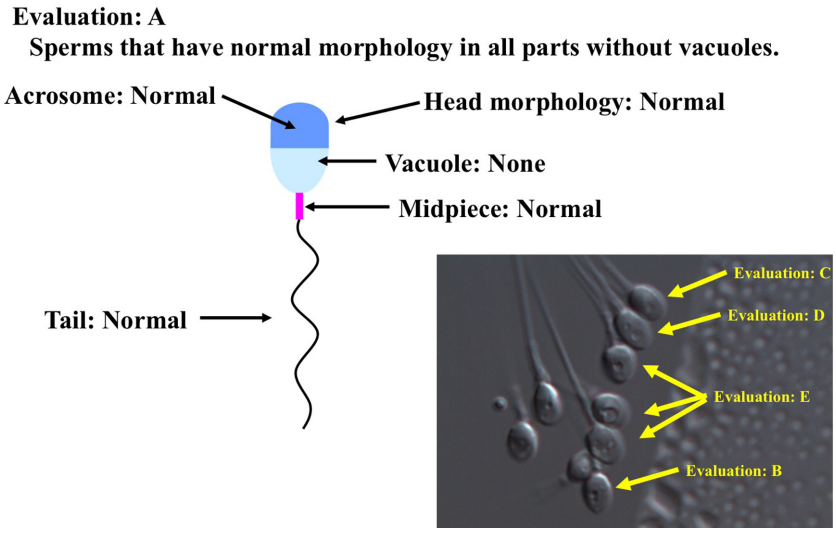

Fig. 4. Evaluation of sperm morphology.

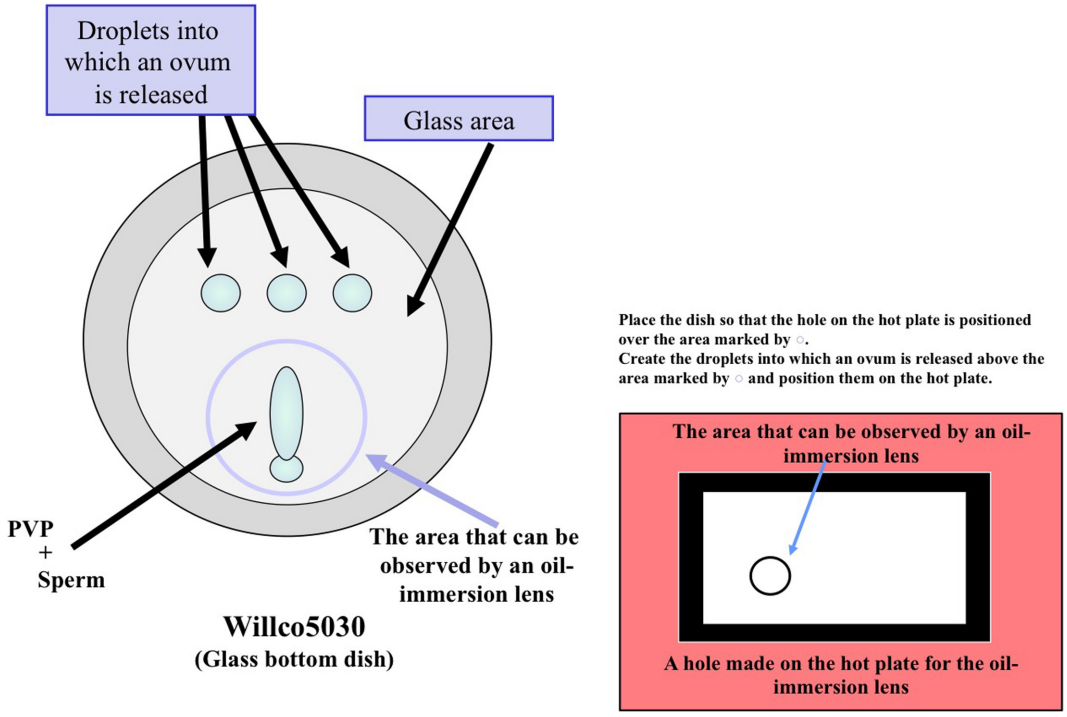

Fig. 5. IMSI procedure.

suspension droplet and the magnification of the objective lens is gradually increased from low magnification to that for a 100 × DIC oil-immersion objective lens. The lever of the 1.6 × intermediate variable magnification lens is pulled to observe the sperms through the eyepiece at 1,600 × magnification. When a sperm evaluated as either A or B is found, it is aspirated by the tail using an injection pipette. Using an image magnified to 6,000–12,500 × by digital zoom processing on a PC monitor, the head morphology and vacuole of the sperm is observed in further detail within the injection pipette. Observation of a sperm in the injection pipette facilitates the morphological evaluation of sperms in a motile state. One of the reasons why sperms are not observed after immobilization is that

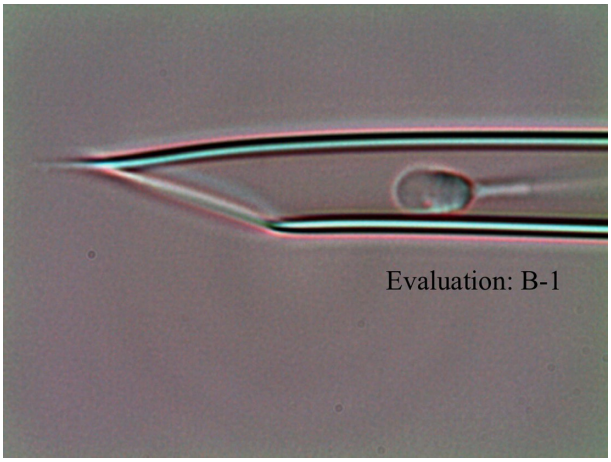

Fig. 6. A sperm inside the injection pipette.

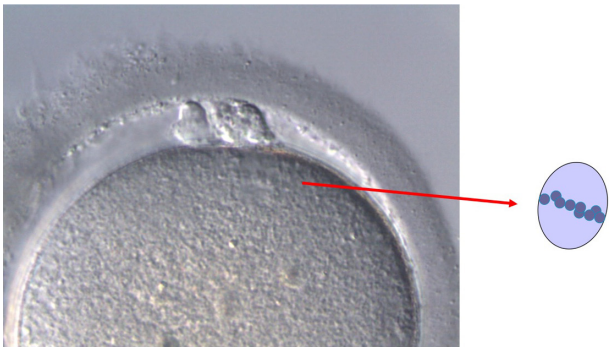

Fig. 8. A spindle observed using 40 × DIC.

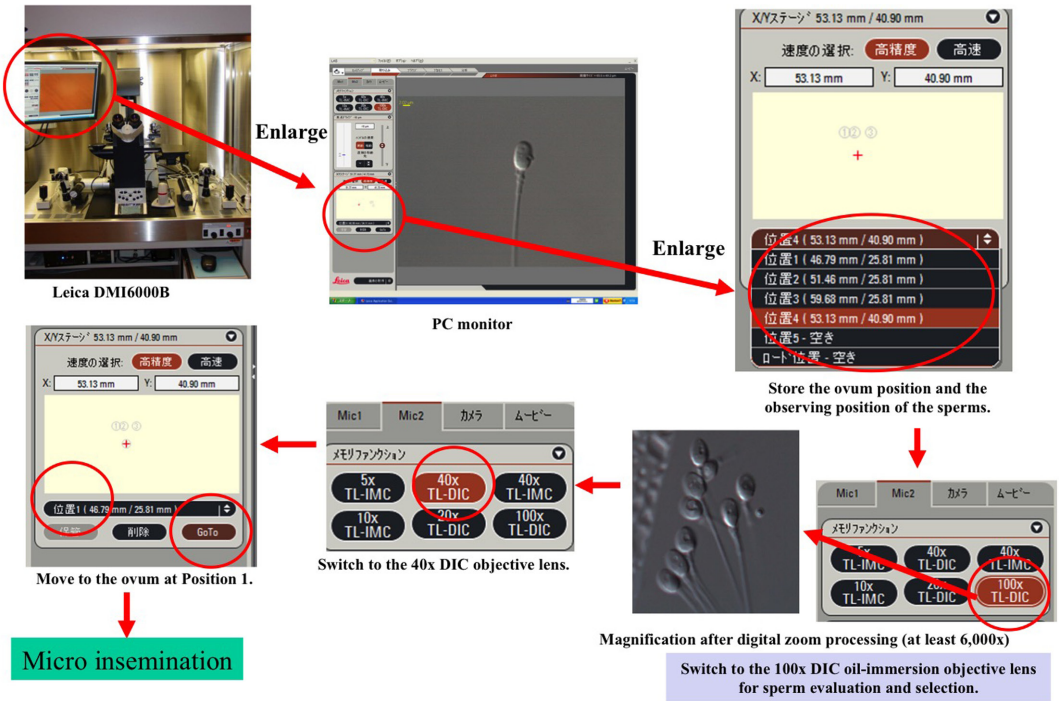

Fig. 7. IMSI procedure.

the outflow of cellular components in the sperm starts upon immobilization, causing the loss of sperm-derived egg-activating factors and making immediate ICSI an imperative. Under these circumstances, it is not appropriate to take the time to observe and examine the sperms (Fig. 6). When it has been decided to use a sperm after a detailed evaluation, the sperm tail is immobilized to stop its movement as is the case with a normal sperm. As the amount of information that can be obtained from observation at a magnification of 6,000 × or higher is orders of magnitude different from that obtained under observation at a magnification of 400 ×, the magnification usually used for ICSI, it takes a certain amount of time to observe morphology. Therefore, it is necessary to contrive ways of observing morphology as long as possible without immobilizing sperms, as the immobilization procedures will greatly affect the final clinical outcome, particularly the fertilization rate. By aspirating the immobilized sperm

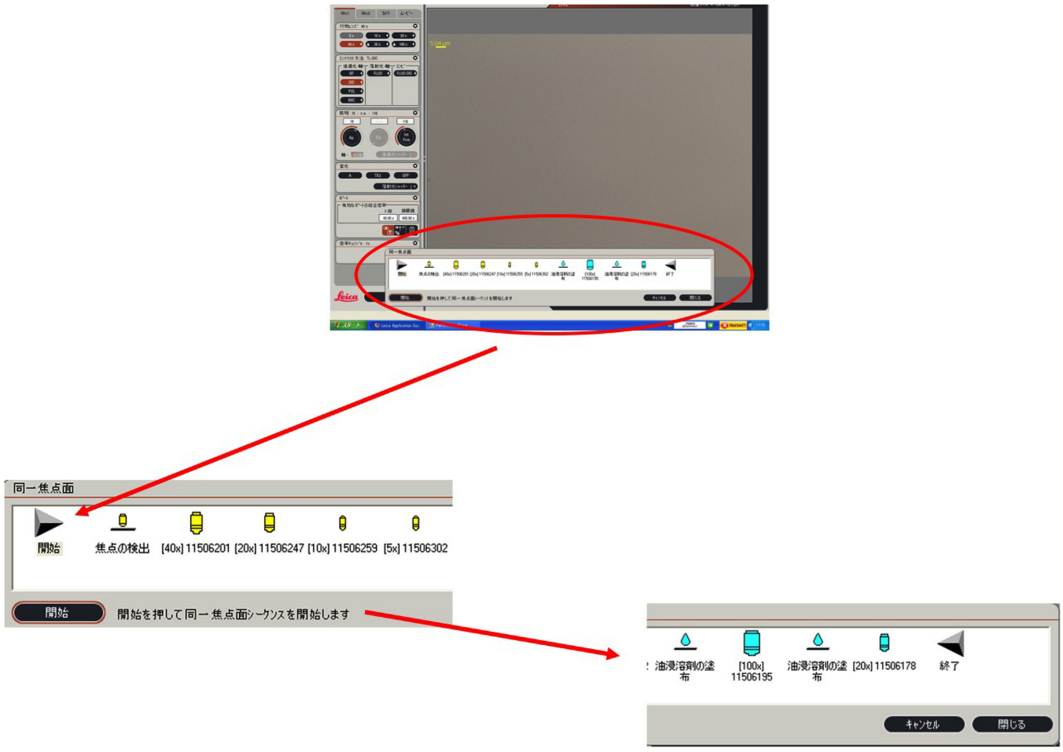

Fig. 9. Setting of the same focal plane.

into an injection pipette again, switching the objective lens to 40 × (DIC), and clicking the position of the first ovum stored in advance on the stage coordinates, the injection pipette can be instantaneously moved to the egg position to start ICSI (micro-insemination) (Fig. 7). ICSI is usually performed by the integrated (Hoffman) modulation contrast (IMC) method using a plastic dish. One of the main reasons why DIC is used for micro insemination is that DIC allows the observation of the spindle in a bright field; thus, offering the benefit of enabling intracytoplasmic sperm injection while reliably avoiding the spindle. DIC was originally introduced to perform IMSI, but is now used to perform ICSI micro-insemination in a safer manner (Fig. 8).

One of the important things for observation of sperm morphology under a high magnification of at least 1,000 × is master the use of the microscope under optimal conditions. This means familiarizing oneself with the adjustment of the microscope as well as the adjustment and maintenance of the camera used for digital zoom processing so that the resolution of the microscope can be fully utilized. Moreover, before performing IMSI, it is desirable to set the same focal plane during calibration before using the microscope. Setting of the same focal plane allows for correction of slight misalignments of the focus between the objective lenses (Fig. 9). The adjustment of the prism is also important observation by DIC. As the contrast is adjusted by the prism, the level of contrast achieved greatly changes how the vacuoles are shown. There are many different types of vacuoles in terms of morphology, such as those that appear to be formed as a result of the fusion of multiple vacuoles, those that appear to be shallow, those that show deep clear contours, and other obscure structures that are difficult to distinguish. Whether or not the microscope can be fully utilized is directly linked to the amount of information that can be obtained from the image, and prompt and accurate evaluation of findings of sperm morphology leads to more accurate sperm selection. Moreover, to master the IMSI technique, it is essential to train operators on a daily basis so that they can perform a series of procedures routinely, including unfamiliar operations performed on a PC.

Results of IMSI

Table 1 shows the revised list of Monte *et al.* [1] that summarises papers regarding IMSI. Bartoov *et al.* [2]

Table 1. Review of the literature

			No of cycles		Fertilization rate (%)			Implantation rate (%)			Pregnancy rate (%)			Abortion rate (%)			High-quality embryos		
			IMSI	ICSI	IMSI	ICSI	*p*	IMSI	ICSI	*p*	IMSI	ICSI	*p*	IMSI	ICSI	*p*	IMSI	ICSI	*p*
Bartoov [2] (2003)	Matching study	At least two previous consequent pregnancy failed ICSI cycle	50	50	64.5 ± 17.5	65.5 ± 21.5	NS	27.9 ± 26.4	9.5 ± 15.3	≤0.01	66	30	≤0.01	9	33	≤0.01	45.2 ± 28.2%	31.0 ± 19.5	≤0.01
Berkovitz [3] (2006)	Matching study	At least two previous consequent pregnancy failed ICSI cycle	80	80	67.4 ± 20.8	69.1 ± 22.6	NS	31.3 ± 36.3	9.4 ± 17.4	≤0.05	60	25	≤0.05	14	40	≤0.05	38.7 ± 31.6%	25.7 ± 28.3	≤0.05
Antinori [4] (2008)	Prospective randomized study	Severe oligoasthenoteratozoospermia	227	219	94.8	94.5	NS	17.3	11.3	0.007	39.2	26.5	0.004	16.9	24.1	NS	-	-	-
Knez [5] (2011)	Prospective randomized study	Poor semen quality with all arrested embryos following a prolonged 5-day culture in previous cycles	20	37	51.2	52.7	NS	17.1	6.8	NS	25	8.1	NS	-	-	-	-	-	-
Setti [6] (2011)	Prospective randomized study	Oligoasthenoteratozoospermia according to the 2010 WHO reference values	250	250	79.2	78.9	NS	23.8	25.4	NS	37.2	36.8	NS	18.4	17.9	NS	44.40%	37.30%	NS
Oliveira [7] (2011)	Prospective randomized study	Repeated implantation failure	100	100	64.5 ± 23.5	62 ± 26.5	NS	13.6	9.8	NS	26	19	NS	15.4	31.6	NS	1.4 ± 0.5 (mean ± SD)	1.5 ± 0.5 (mean ± SD)	NS
Balaboan [8] (2011)	Prospective randomized study	Unselected infertile population	87	81	81.60 ± 10.65	80.97 ± 15.06	NS	28.9	19.5	NS	54	44.4	NS	-	-	-	-	-	-
Marci [9] (2013)	Prospective randomized study	Unselected infertile population	51	281	80	77.27	NS	16.67	16.83	NS	33.33	30.96	NS	5.26	17.78	NS	-	-	-
Leandri [10] (2013)	Prospective randomized study	First ART attempt for male infertility	116	139	56	63	<0.05	24	23	NS	31	33	NS	-	-		-	-	-

Revised list by Monte *et al.* [1]. Abbreviation: NS, not significant; IMSI, Intracytoplasmic Morphologically-selected Sperm Injection; ICSI, Intracytoplasmic Sperm Injection.

originally reported the efficacy of selecting sperms at high magnification. In their paper, they presented the technique “motile sperm organelle morphology examination (MSOME)”, and named the method in which sperms are microscopically selected and fertilized at high magnification as “intracytoplasmic morphologically selected sperm injection (IMSI)”. The subjects of their study were 50 couples treated with IMSI, who had at least two previous ICSI cycle failures, and 50 matched couples treated with normal ICSI. A comparison of IMSI (in which sperms with completely normal morphology, those with normal morphology confined to the head parts, and those with vacuoles in the head parts are selected for use, based on the observation of acrosome, post-acrosomal lamina, nucleus [shape, chromatin content], neck, and tail mitochondria after eliminating abnormal sperms under observation at 400 × magnification) and ICSI (in which sperms are selected under observation at 400 × magnification) showed there was no significant difference between the fertilization rates (IMSI, 64.5%; ICSI, 65.5%), while significantly higher rates of top embryo (IMSI, 45.2%; ICSI, 31.0%), implantation (IMSI, 27.9%; ICSI, 9.5%), and pregnancy (IMSI, 66%, ICSI, 30%) were observed in IMSI, and an abortion rate of IMSI was significantly lower than that of ICSI (IMSI, 9%; ICSI, 33%).

Similar to Bartoov *et al.*, Berkovitz *et al.* [3] (from the same research group) also reported that there was no significant difference between the fertilization rates of IMSI and ICSI, while significantly higher rates of top embryo, implantation, and pregnancy were observed in IMSI, and a significantly lower abortion rate than that of ICSI. Their subjects were another 80 couples treated with IMSI, who had at least two previous ICSI cycle failures, and 80 matched couples treated with normal ICSI. Berkovitz *et al.* also compared the results of 70 cycles of IMSI, in which the best sperm rated as completely normal with MSOME was selected and used, and those of the 70 cycles of IMSI, in which the second best sperm was selected and used, because no sperm that could be rated as completely normal was found due to small vacuoles etc. in the head parts of sperms. They reported that the fertilization rate, the ratio of top embryo, the implantation rate, the pregnancy rate, and the delivery rate were significantly higher in the 70 cycles with the best sperm as compared to those with the second best sperm, while the abortion rate was significantly lower.

Antinori *et al.* [4] performed 227 IMSI cycles and 219 ICSI cycles for 446 couples with severe oligoasthenozoospermia and compared the results. The implantation rates were 17.3% (IMSI) and 11.3% (ICSI), and the pregnancy rates were 39.2% (IMSI) and 26.5% (ICSI). Both the implantation rate and the pregnancy rate of IMSI were significantly higher than those of ICSI. They also reported that the abortion rates were 16.9% in IMSI and 24.1% in ICSI, and the abortion rate of IMSI was significantly lower than that of ICSI. Furthermore, couples with at least two pregnancy failures after ART showed a significantly higher pregnancy rate after IMSI than after ICSI (IMSI, 29.9%; ICSI, 12.9%), while the abortion rate of IMSI was significantly lower (IMSI, 17.4%; ICSI, 37.5%).

Knez *et al.* [5] compared the results of IMSI with those of ICSI for couples whose embryos had not differentiated into blastocysts and arrested due to the poor semen quality despite prolonged 5-day culture in earlier ICSI cycles. They reported that no significant differences were observed between IMSI and ICSI in the fertilization, implantation, and pregnancy rates, although the rates at which all embryos arrested again after long-term culture were 0% (IMSI) and 27.0% (ICSI), the rate of IMSI being significantly lower.

In the first cycle of *in vitro* fertilization (IVF) for 500 couples with oligoasthenozoospermia, determined according to the criteria of semen analysis specified by WHO in 2010, Setti *et al.* [6] prospectively compared the results of 250 IMSI cycles with those of 250 ICSI cycles. They reported that the normal fertilization rates were 79.2% in IMSI and 78.9% in ICSI, the high-quality embryo rates were 44.4% in IMSI and 37.3% in ICSI, the implantation rates were 23.8% in IMSI and 25.4% in ICSI, the pregnancy rates were 37.2% in IMSI and 36.8% in ICSI, and the abortion rates were 18.4% in IMSI and 17.9% in ICSI, with no significant differences between IMSI and ICSI for any of them.

Oliveira *et al.* [7] reported that a comparison of the results of IMSI and ICSI for women with repeated implantation failure revealed that, although IMSI tends to be better than ICSI, there were no significant differences between IMSI and ICSI in fertilization rate (IMSI: 65.4%, ICSI: 62.0%), implantation rate (IMSI: 13.6%, ICSI: 9.8%), pregnancy rate (IMSI: 26%, ICSI: 19%) or live birth rate (IMSI: 21%, ICSI: 12%).

Balaban *et al.* [8] performed a prospective comparison of the results of IMSI cycles (n=87) and ICSI cycles (n=81) for an unselected infertile population. They reported the results of normal fertilization rate (IMSI: 81.60%, ICSI: 80.97%), high-quality embryo rate (IMSI: 66.44%, ICSI: 63.95%), implantation rate (IMSI: 28.9%, ICSI: 19.5%), pregnancy rate (IMSI: 54.0%, ICSI: 44.4%) and live birth rate (IMSI: 43.7%, ICSI: 38.3%) showed no significant differences between IMSI and ICSI. They also reported, that when limited to couples with severe male factor infertility, the implantation rate of IMSI was significantly

higher than that of ICSI (IMSI: 29.6%, ICSI: 15.2%).

Marci *et al.* [9] performed a prospective comparison of the results of IMSI cycles (n=51) and ICSI cycles (n=281) for an unselected infertile patient population. They reported the results of fertilization rate (IMSI: 80.00%, ICSI: 77.27%), implantation rate (IMSI: 16.67%, ICSI: 16.83%), pregnancy rate (IMSI: 33.33%, ICSI: 30.96%), abortion rate (IMSI: 5.26%, ICSI: 17.78%) and live birth rate (IMSI: 13.72%, ICSI: 11.39%) showed no significant differences between IMSI and ICSI. They also reported that when cases were limited to male factor infertility or repeated IVF failure the results of IMSI and of ICSI still showed no significant differences.

Leandri *et al.* [10] performed a multicentric randomized controlled trial for the first ART cyle for male factor infertility, and reported that in 116 IMSI cycles and 139 ICSI cycles performed for cases with sperm counts after preparation of 1 million or less, the fertilization rate of IMSI was significantly lower (IMSI: 56%, ICSI: 63%). The results for implantation rate (IMSI: 24%, ICSI: 23%), clinical pregnancy rate (IMSI: 31%, ICSI: 33%) and delivery rate (IMSI: 27%, ICSI: 30%) showed no significant differences between IMSI and ICSI.

Berkovitz *et al.* [11] performed a comparison of IMSI outcomes, using sperm which is normal except for a head with a large vacuole and IMSI outcomes using normal sperm with no vacuole in the head. Although no differences were observed between the groups in terms of fertilization or high-quality embryo rate, the group with a sperm head with a large vacuole showed a significantly lower pregnancy rate, 18% than that of the group with a sperm head with no vacuole 50%. Regarding the abortion rate, the group with a sperm head with a large vacuole showed a significantly higher rate, 80%, than that of the group with a sperm head with no vacuole, 7%.

Hazout *et al.* [12] performed IMSI for 125 couples with at least two pregnancy failures in normal ICSI, and compared the outcomes with those of the previous ICSI cycle. They reported there were no changes in fertilization rate, cleavage rate or high-quality embryo rate, though the pregnancy rate and the implantation rate significantly increased after IMSI. In addition, from the viewpoint of the sperm DNA fragmentation rate evaluated by the TUNEL method, practice of IMSI has led to significant increases in implantation rates and the delivery rates, compared to the previous ICSI cycle, at rates of sperm DNA fragmentation of no more than 30%, from 30 to 40%, and at no less than 40%.

Greco *et al.* [13] retrospectively studied the impact of vacuoles in sperm with impaired sperm morphology of no more than 3% in the Krueger test. IMSI for 101 couples with at least twice the normal ICSI failure rate. A comparison of IMSI outcomes between good quality sperm and low quality sperm found no differences in the parameters of fertilization rate (good quality sperm: 73.7%, low quality sperm: 66.9%) and rate of top quality embryo (good quality sperm: 84.0%, low quality sperm: 84.7%). However, as seen in the results of the clinical pregnancy rate (good quality sperm: 41.7%, low quality sperm: 17.1%), implantation rate (group for good quality sperm: 23.1%, group for low quality sperm: 17.1%) and live birth rate (good quality sperm: 36.7%, low quality sperm: 14.3%), the results of good quality sperm were better.

Cassuto *et al.* [14] have reported that the major malformation rate for children born through micro- insemination was significantly lower, 1.33%, in IMSI than in ICSI, 3.80%.

Conclusion

Since the establishment of intracytoplasmic sperm injection in 1992, sperms have been observed, evaluated, and selected under an inverted microscope at 400 × magnification in ICSI. Intracytoplasmic morphologically selected sperm injection is a technique in which sperms are selected and used for micro-insemination by more precisely observing their morphology at increased magnification under an inverted microscope used for micro-insemination and enhancing the image resolution. Approximately 20 years have passed since IMSI was developed, and until now its efficacy has only been confirmed the cases with a high rate of sperm aneuploidy, a high rate of sperm DNA fragmentation, or repeated ICSI failure. It is well-known that the quality of sperms has a profound effect on the quality of embryos, which may eventually become children. Therefore, when micro insemination is performed, sperms should be selected by not only paying attention to their motility, but also by closely observing their morphology. Those engaged in fertility treatment should always keep in mind that sperm selection itself has a significant influence on patients' lives.

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