

Article

Intracytoplasmic morphologically selected sperm injection: a prospective randomized trial



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Abstract

The aim of this prospective randomized study was to assess the advantages of a new modified intracytoplasmic sperm injection (ICSI) technique called intracytoplasmic morphologically selected sperm injection (IMSI) over the conventional ICSI procedure in the treatment of patients with severe oligoasthenoteratozoospermia. The new procedure consisted of IMSI based on a preliminary motile sperm organellar morphology examination under $\times 6600$ high magnification. A total of 446 couples with at least two previous diagnoses of severe oligoasthenoteratozoospermia, 3 years of primary infertility, the woman aged 35 years or younger, and an undetected female factor were randomized to IVF micro-insemination treatments: ICSI ($n = 219$; group 1) and IMSI ($n = 227$; group 2). A comparison between the two different techniques was made in terms of pregnancy, miscarriage and implantation rates. The data showed that IMSI resulted in a higher clinical pregnancy rate (39.2% versus 26.5%; $P = 0.004$) than ICSI when applied to severe male infertility cases. Despite their initial poor reproductive prognosis, patients with two or more previous failed attempts benefited the most from IMSI in terms of pregnancy (29.8% versus 12.9%; $P = 0.017$) and miscarriage rates (17.4% versus 37.5%). At present, 35 healthy babies have been born following the introduction of this promising technique in daily IVF practice.

Keywords: high magnification, ICSI, IMSI, male infertility, MSOME, sperm selection

Introduction

Sperm morphology evaluation plays a crucial role in the diagnosis of male fertility potential and it has demonstrated a predictive value for fertilization and pregnancy outcomes in IVF treatments (Kruger *et al.*, 1986, 1987; Parinaud *et al.*, 1993; Ombelet *et al.*, 1997; Eilish *et al.*, 1998). A different prognosis can be assigned on the basis of different normal morphology thresholds (poor prognosis: $\leq 4\%$; good prognosis: 5–14%; normal: $>14\%$) in order to choose an adequate infertility management (Kruger *et al.*, 1988; Grow *et al.*, 1994). Following the introduction of micro-insemination techniques in humans (Ng *et al.*, 1988; Fishel *et al.*, 1990; Palermo *et al.*, 1992; Antinori *et al.*, 1995) sperm morphology evaluation has lost its exclusive diagnostic role to become an active part of the laboratory procedure once the embryologist has begun to

select a motile, normal-looking spermatozoon to be injected into the oocyte under $\times 200/400$ magnification. Sperm selection is based on the judgment of an embryologist who selects the most normal-looking spermatozoon available under $\times 200/400$ magnification (**Figure 1**). Several authors have shown how the correct selection of spermatozoa improves intracytoplasmic sperm injection (ICSI) outcome (Kahraman *et al.*, 1999; Miller and Smith, 2001; De Vos *et al.*, 2003).

Because of the subjective nature of this evaluation, several instruments for computerized sperm morphology analysis have been developed (Davis *et al.*, 1992; Kruger *et al.*, 1995; Lau and Chalmers, 1995; Sukcharoen *et al.*, 1998), and recently new devices to achieve high magnification levels have been

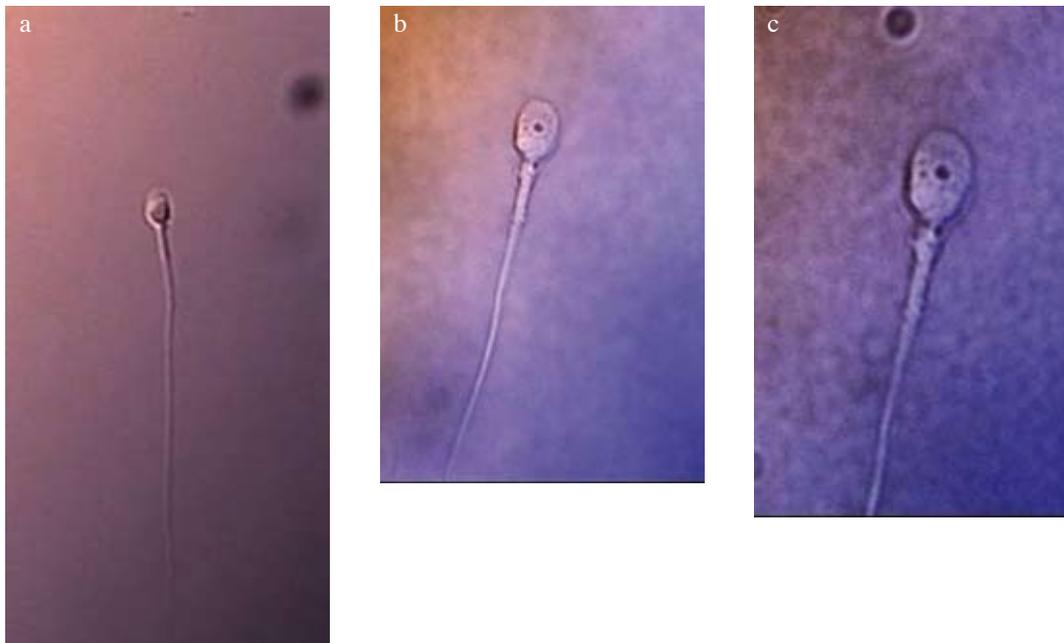


Figure 1. A human spermatozoon: (a) $\times 400$, apparently suitable for intracytoplasmic sperm injection procedure; (b) $\times 3670$, the same spermatozoon showing head (vacuoles, vesicles, irregular postlamina) and neck malformations; (c) $\times 5880$, a close-up of the previous malformations.

proposed in order to detect subtle ultra-structural alterations that would be impossible to identify with conventional methods.

A new method of high magnification ($\times 6600$) motile sperm organellar morphology examination (MSOME) published by Bartoov *et al.* in 2002 showed a positive and significant correlation between the incidence of morphologically normal spermatozoa and fertilization rate following ICSI.

Out of six sperm subcellular organelles examined (neck, tail, mitochondria, acrosome, postacrosomal lamina, nucleus) the morphological normalcy of the sperm nucleus (shape, chromatin content) was significantly and positively associated with both fertilization rate and pregnancy outcome. In 2003, Bartoov used MSOME criteria to select single motile spermatozoa for ICSI (a technique called intracytoplasmic morphologically selected sperm injection, IMSI) in patients with at least two previous ICSI failures. The patients who underwent IMSI obtained significantly better results in terms of pregnancy and miscarriage rates. Later, based again on the MSOME criteria, Hazout applied high magnification techniques ($\times 6000$) to the sperm selection process for the ICSI procedure, showing a significant improvement in clinical IVF outcome in patients with previous failed ICSI attempts (Hazout *et al.*, 2006). These encouraging results offer a new perspective for future improvements in assisted reproduction techniques.

Both of the above studies restricted the application of this new procedure to cases with more than two previous implantation failures. Actually, according to recent publications, these couples seem to have the worst reproductive prognosis,

exhibiting a dramatic reduction in pregnancy and implantation rates as against couples with no or one previous failed IVF attempt (Shapiro *et al.*, 2001; Silberstein *et al.*, 2005).

Unfortunately in 2004 a new law on assisted reproduction was enacted in Italy that limits the number of oocytes that can be fertilized to three, and in some cases this seems to have decreased the chances for those couples seeking pregnancy through IVF (Pellegrini *et al.*, 2005; Greco *et al.*, 2006; Ciriminna *et al.*, 2007).

The main purpose of this study was to assess the potential advantages of the IMSI procedure in the treatment of patients with severe oligoasthenoteratozoospermia regardless of their previous failed ICSI attempts, based on a prospective randomized controlled protocol, in the context of further standardization and wider application of the method.

Following subgroup splitting according to the number of previous failed attempts, a secondary data analysis was performed to investigate the usefulness of the IMSI procedure for those categories (less than two previous failed attempts) that previous studies had excluded a priori from the application.

Materials and methods

Patient selection

A preliminary pilot study was made on 30 cases (15 cases for each technique) giving pregnancy rates of 28.5% and 14% for IMSI and ICSI techniques, respectively. On the basis of these

clinical results, a trial with a power of 90%, an α value of 0.05 and a 5% of potential unperformed transfer (caused by missed fertilization or cleavage) would require a total sample size of 361 patients. At that time it was not known whether and how couples could be classified into subgroups based on the previous failure number.

From January 2006 up to June 2007, 446 couples were enrolled. Admission criteria included: (i) at least two previous diagnoses of severe oligoasthenoteratozoospermia, (ii) at least 3 years of primary infertility, (iii) the woman being 35 years or younger, (iv) an undetected female factor. After having obtained the patients' informed consent, couples were randomly allocated to receive the IVF micro-insemination treatments using computer-generated random numbers (randomization list without any restriction), concealed in sealed, opaque envelopes prepared by a research nurse.

All study participants were blinded to treatment assignment for the duration of the study. The following groups were formed: ICSI ($n = 219$; group 1) and IMSI ($n = 227$; group 2).

After this randomization, based on the number of previous failed ICSI attempts, three subgroups were identified for each technique: group A, no previous attempts; group B, one previous failed attempt; group C, two or more previous failed attempts. Hence, patients were divided as follows: sub-group 1A ($n = 50$), sub-group 1B ($n = 107$), sub-group 1C ($n = 62$), sub-group 2A ($n = 73$), sub-group 2B ($n = 77$), sub-group 2C ($n = 77$).

Semen evaluation and preparation

The diagnosis of severe oligoasthenoteratozoospermia was confirmed by the World Health Organization (WHO) criteria for sperm concentration ($<5 \times 10^6/\text{ml}$) and motility ($<20\%$ progressive) (WHO, 1992), and strict criteria ($<4\%$ normal forms) for sperm morphology evaluation (Kruger *et al.*, 1988), resulting in (mean \pm SD): $2.6 \pm 0.4\%$, and $2.3 \pm 0.3\%$ for ICSI and IMSI groups, respectively.

Only freshly ejaculated semen was used for this study.

The preparation of the semen was performed on the basis of a two-layer (low density and high density) SIL-Select density gradient system using HEPES/Ham's F-10 supplemented with 20% serum substitute supplement (Irvine Scientific, Santa Ana, CA, USA).

IMSI sperm selection

The Petri dish (Willco-dish; Willco wells BV, Amsterdam, The Netherlands) for IMSI was prepared as follows: (i) on the left side, three observation droplets of $4 \mu\text{l}$ each, made up with polyvinylpyrrolidone medium (MediCult, Jyllinge, Denmark) at decreasing concentrations (undiluted, 3% and 0%) in order to make a balance between solution toxicity and sperm motility preservation; (ii) in the middle, one $4 \mu\text{l}$ droplet (selection droplet) of HEPES/Ham's F-10 medium supplemented with 20% serum substitute supplement to store selected sperm cells; (iii) on the right side, one to three (depending on the number of oocytes available) $4 \mu\text{l}$ droplets (injection droplets) of HEPES/Ham's F-10 supplemented with 20% serum substitute supplement, to

host the oocytes that were to be injected by the following ICSI procedure.

All microdroplets were placed under sterile liquid paraffin (MediCult).

The sperm cell suspension obtained after semen preparation was used for real-time high magnification MSOME (Bartoov *et al.*, 2003) that was performed on the observation droplets by means of an inverted microscope (Olympus IX81, Tokyo, Japan) equipped with Nomarski differential interference contrast optics, an Uplan Apo $\times 100$ oil/1.50 objective lens, and a 0.55 numerical aperture condenser lens. The images were captured by a DXC-990P colour video camera (Sony) having 0.5 inch, three-chip power HAD CCD (total calculated magnification $\times 6600$) and visualized on a monitor screen with diagonal dimension of 355.6 mm.

Spermatozoa with severe malformations, such as a pin, amorphous, tapered, round or multinucleated head, which can be identified clearly even by low magnification ($\times 200$ to $\times 400$), were not assessed by MSOME (Berkovitz *et al.*, 2006a).

In order to perform a correct sperm evaluation, the embryologists have to follow each apparently suitable single sperm cell by moving the microscopic stage in the x, y and z directions until they observe even the smallest details. Spermatozoa with abnormal head size were excluded by superimposing on the motile examined gametes a transparent celluloid form, representing the correct sperm size, which was calculated by the ratio of normal expected sperm size to the actual size visualized on the monitor screen. The sperm selection procedure does not require any computer application since the automated sperm morphology analysis systems available on the market allow only morphology evaluation of immotile spermatozoa on stained slides.

Only motile spermatozoa with normal head dimensions (length $4.75 \pm 0.28 \mu\text{m}$, width $3.28 \pm 0.20 \mu\text{m}$) and shapes (Bartoov *et al.*, 2002), and with no or a maximum of one vacuole ($0.78 \pm 0.18 \mu\text{m}$) were retrieved from the observation droplets and aspirated into a sterilized glass non-angled pipette with a $9 \mu\text{m}$ inner diameter tip (Humagen, Charlottesville, VA, USA). Sperm cells were then placed into the selection droplet and finally were used for injection into the oocytes by classical ICSI (Palermo *et al.*, 1992). This procedure was performed using an Eppendorf Micromanipulation System (TransferMan NK2; Eppendorf Germany). Spermatozoa with a doubtful determination were excluded from selection. Two spermatozoa for each oocyte to be inseminated were selected (maximum three oocytes for each patient, in compliance with the Italian law).

Finding normal-looking spermatozoa took a minimum of 60 min, and up to 210 min, depending on the quality of the semen sample in each case. The technique required two embryologists working together on the analysis of the same sample at the same time in order to minimize the subjective nature of sperm evaluation.

Ovarian stimulation, oocyte retrieval and culture protocol

Ovarian stimulation was achieved using a gonadotrophin releasing hormone antagonist, ganirelix acetate (Orgalutran; Organon Inc., Italy), in association with recombinant human

FSH (Gonal F; Serono, Italy), 150 IU per day. Follicular growth was monitored by serum 17β -oestradiol measurements and ovarian ultra-sonography. Ovulation was induced by the administration of 10,000 IU human chorionic gonadotrophin (Gonasi HP; AMSA, Italy) when at least three follicles of diameter ≥ 17 mm were observed and with 17β -oestradiol concentrations corresponding to the number of follicles. Transvaginal, ultrasound-guided oocyte retrieval was performed 34–36 h later. After retrieval, three of the recovered oocytes were transferred to the dishes containing the culture medium (MediCult, Italy) and submitted to the ICSI procedure. All oocytes used for ICSI were mature at the time of injection and had normal morphology (Veeck, 1991).

Progesterone (Prontogest; AMSA) administration (50 mg daily) was started 72 h prior to the embryo transfer. All couples included in the present study had at least one embryo to be transferred. An endometrial thickness of at least 8 mm was considered suitable for embryo transfer. The embryo quality was scored according to Steer *et al.* (1992). The clinician who performed the embryo transfer and administered the following supporting therapy was blinded to the treatment allocation.

The primary outcome measures were clinical pregnancy, miscarriage and implantation rates. Clinical pregnancy was defined as a positive β -human chorionic gonadotrophin (β -HCG) assay and the presence of at least one gestational sac with fetal heartbeat detection by transvaginal ultrasound examination. Miscarriage was defined as pregnancy loss before 24 weeks gestation. Implantation rate was defined as the total number of gestational sacs presenting heart pulsations in relation to the total number of embryos transferred.

Statistical analysis

Student's *t*-test was used to compare continuous variables, whereas a chi-squared test was applied to discrete variables. $P < 0.05$ was considered statistically significant.

Results

There were no statistical differences between the main groups in terms of mean age, number of previous failed ICSI attempts, number of recovered oocytes and transferred embryos (**Table 1**).

A positive β -HCG test followed by fetal heartbeat detection occurred in 89 IMSI and in 58 ICSI patients. Up to now, the IMSI procedure has resulted in 27 deliveries of a total of 35 healthy babies (eight twins), 47 ongoing pregnancies and 15 miscarriages. As for the ICSI group, 25 healthy babies were born (one twin), 14 miscarriages occurred and 20 pregnancies are still ongoing. All miscarriages took place during the first trimester.

By comparing groups 1 and 2, IMSI pregnancy and implantation rates appear to be significantly higher than those for ICSI (pregnancy rate 39.2% versus 26.5%, $P = 0.004$; and implantation rate 17.3% versus 11.3%, $P = 0.007$).

Upon comparison of the two techniques by subgroups with different previous failed attempts (**Table 2**), the following pregnancy rate results were obtained: (i) sub-group 1C versus sub-group 2C: 12.9% versus 29.9%, $P = 0.017$; (ii) no statistical difference was observed between sub-group 1A and sub-group 2A (28.0% versus 42.5%), and between sub-group 1B and sub-group 2B (33.6% versus 45.5%) (**Table 2**), although the clinical outcome was clearly in favour of the IMSI method. Furthermore, by combining sub-groups A and B for each technique, which individually revealed no statistically significant differences in their reproductive outcomes, two new sub-groups were formed (2A + 2B = sub-group 2D; 1A + 1B = sub-group 1D) for comparison resulting in pregnancy rates of 44.0% versus 31.8%, $P = 0.028$.

No statistical differences in miscarriage rates for the various groups and sub-groups were observed even if a clear clinical

Table 1. Comparison of fertilization, pregnancy, implantation and miscarriage rates arising from intracytoplasmic sperm injection (ICSI) and intracytoplasmic morphologically selected sperm injection (IMSI) groups.

	Group 1, ICSI (n = 219)	Group 2, IMSI (n = 227)
Mean age (years)	31.91 \pm 3.30	31.65 \pm 3.23
No. of previous ICSI failures	1.45 \pm 1.57	1.46 \pm 1.69
No. of MII oocytes recovered	6.29 \pm 3.04	6.58 \pm 3.34
No. of injected oocytes	2.92 \pm 0.26	2.90 \pm 0.29
No. of two PN zygotes	2.76 \pm 0.42	2.75 \pm 0.45
No. of transferred embryos/patient	2.37 \pm 0.67	2.47 \pm 0.68
Clinical pregnancy rate (%)	58/219 (26.5) ^a	89/227 (39.2) ^a
Implantation rate (%)	59/521 (11.3) ^b ; 1 twin	97/560 (17.3) ^b ; 8 twins
Miscarriage rate (%)	14/58 (24.1)	15/89 (16.9)

MI = metaphase II; PN = pronucleate. Continuous variables are presented as means \pm SD.

^a $P = 0.004$; ^b $P = 0.007$.

Table 2. Comparison of pregnancy and miscarriage rates arising from intracytoplasmic sperm injection (ICSI) and intracytoplasmic morphologically selected sperm injection (IMSI) sub-groups with a different number of previous IVF failures.

Sub-group	Rate	Group 1, ICSI	Group 2, IMSI
Sub-group A (0 IVF failures)	Pregnancy	28.0 (14/ 50)	42.5 (31/73)
	Miscarriage	21.4 (3/14)	12.9 (4/31)
Sub-group B (1 IVF failure)	Pregnancy	33.6 (36/107)	45.5 (35/77)
	Miscarriage	22.2 (8/36)	20.0 (7/35)
Sub-group C (≥ 2 IVF failures)	Pregnancy	12.9 ^a (8/62)	29.9 ^a (23/77)
	Miscarriage	37.5 (3/8)	17.4 (4/23)

Values are percentages with numbers in parentheses.

^a $P = 0.017$.

trend towards the IMSI procedure was seen (**Tables 1 and 2**; subgroup D: IMSI = 16.6%, ICSI = 22.0%).

Discussion

According to some authors, ICSI outcome is not related to strict morphology of the spermatozoon used for microinjection (Oehninger *et al.*, 1995; Kupker *et al.*, 1998; Host *et al.*, 2001; Celik-Ozenci *et al.*, 2004). No differences in terms of fertilization and clinical pregnancy rates have been shown when samples with poor morphology (<5% normal cells) were used as if the only requirement for achieving satisfactory results were the presence of a living, motile spermatozoon (Gomez, 2000).

Furthermore, fertilization, embryo development and pregnancy seem to be achievable even if normal spermatozoa are not available (100% of terato-zoospermia) (Nagy *et al.*, 1995; Tasdemir *et al.*, 1997; McKenzie *et al.*, 2004). On the other hand, some trials have shown low fertilization, pregnancy and implantation rates when spermatozoa with severe anomalies were used for ICSI (Kahraman *et al.*, 1999; De Vos *et al.*, 2003). Moreover, in 2001 Miller and Smith compared ICSI and IVF and embryo transfer cases and revealed that sperm morphology is significantly correlated to the percentage of embryos developing to the blastocyst stage (30.3% versus 51.9%) and to high quality blastocysts (13.6% versus 28.2%).

Later studies reported new opportunities to treat severe male infertility by using micro-insemination procedures made under high magnification systems (Bartoov *et al.*, 2003; Berkovitz *et al.*, 2005). Following injection of spermatozoa without nuclear alterations into oocytes from couples with at least two previous ICSI failures and an undetected female infertility factor, the pregnancy rate doubled, and the miscarriage rate was reduced by 50% as against similar cases treated by conventional ICSI (Berkovitz *et al.*, 2005, 2006a; Hazout *et al.*, 2005).

In order to assess these results and investigate future applications of this method, a prospective randomized trial was designed that involved patients with severe oligoasthenoteratozoospermia regardless of previous ICSI failures. Based on a first overview

of all 446 treated cases, a poor reproductive prognosis could be assigned to the sub-groups with two or more failures having a pregnancy rate of 22.3%, which was significantly lower ($P = 0.005$) than 36.6% and 38.6% scored by patients with none and one previous failure, respectively (**Table 3**) as shown by other authors (Shapiro *et al.*, 2001; Silberstein *et al.*, 2005).

As far as the reproductive outcome is concerned, IMSI resulted in a significantly higher pregnancy rate than ICSI in all treated cases ($P = 0.004$) and notably in patients with two or more failures for whom the success rate increased by over 100% ($P = 0.017$), which confirms the data already published in the literature (Bartoov *et al.*, 2003; Berkovitz *et al.*, 2005, 2006a,b; Hazout *et al.*, 2005).

It is likely that in those couples the male factor could be the result of semen impairment, undetected by conventional diagnostic tools, thus reducing the effectiveness of previous ICSI treatments (**Figures 1 and 2**). The above comparisons did not show any statistical difference in terms of miscarriages but the clinical trend seems to support the IMSI method with a remarkable 50% reduction in the miscarriage rate in subgroup 2C versus subgroup 1C (17% and 37%, respectively). In this respect, it should be added that at the time of writing, about half of ICSI and IMSI pregnancies are still ongoing; furthermore, the comparison between IMSI and ICSI in the three subgroups was clearly affected by the number of cases, since subgroup splitting reduced the sample size, preventing statistical significance from being reached. Thus, the better results in terms of pregnancy and miscarriage rates following IMSI in couples with no or one previous IVF failure are of interest, although further investigations are needed to enlarge the sample size.

The results seem to be even more valuable considering the limited number of fertilizable oocytes imposed by the Italian law (maximum three), which reduces the number and quality of the embryos suitable for transfer. A strong need to increase efficiency of micro-insemination techniques offers a decided thrust to any improvement that could help overcome law restrictions in Italy. As to the advances introduced by IMSI, this technique requires some special equipment to reach the necessary magnification (microscope, camcorder, composite

Table 3. Comparison of pregnancy and miscarriage rates arising from sub-groups with a different number of previous IVF failures in the entire study population.

	Sub-group A (0 IVF failures)	Sub-group B (1 IVF failure)	Sub-group C (≥2 failures)
Pregnancy rate	36.6 ^a (45/123)	38.6 ^a (71/184)	22.3 ^b (31/139)
Miscarriage rate	15.6 (7/45)	21.1 (15/71)	22.6 (7/31)

Values are percentages with numbers in parentheses.
For a versus b: $P = 0.005$.

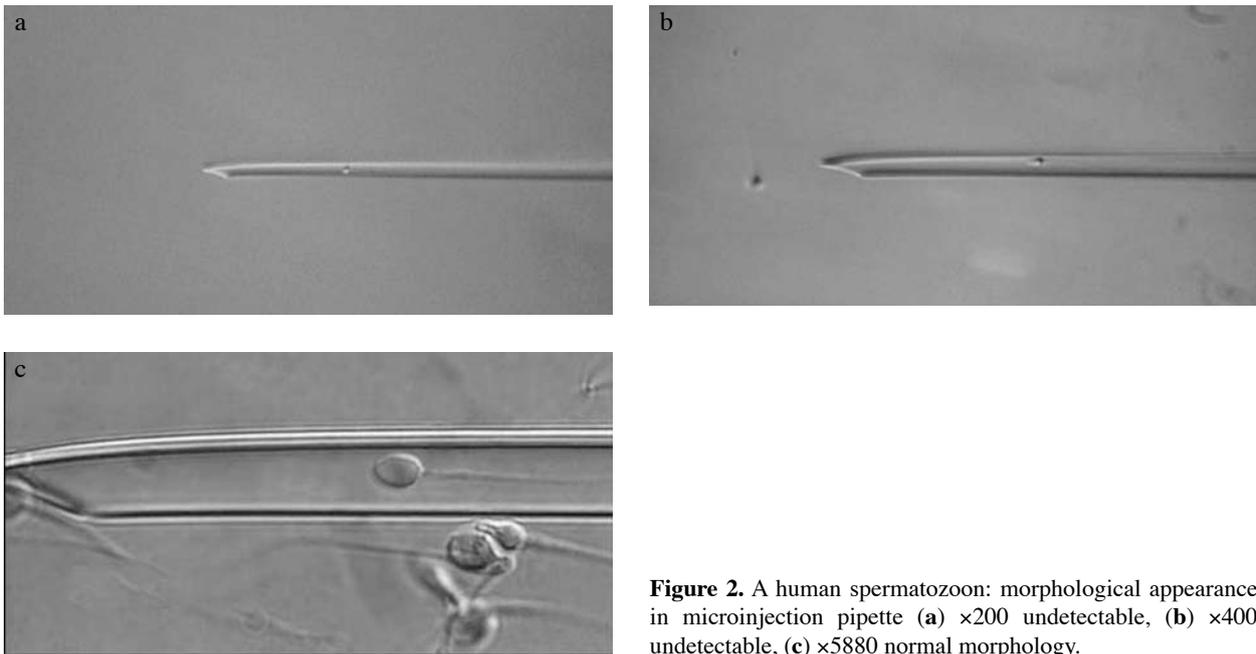


Figure 2. A human spermatozoon: morphological appearance in microinjection pipette (a) ×200 undetectable, (b) ×400 undetectable, (c) ×5880 normal morphology.

system of lenses), and useful criteria (MSOME; Bartoov *et al.*, 2002) for the correct selection of male gametes, but above all it has to be performed by experienced, highly qualified embryologists.

Furthermore, it is relevant to emphasize how a single sperm evaluation is reliable only when it is carried out on a motile sperm cell; static sperm images allow only evaluation of the visible part, leaving some morphological alterations undiscovered.

The extra time necessary for a correct selection and the need to work in pairs to increase accuracy of the evaluation are still a real limit to a more widespread use of this technique.

In conclusion, to the best of the authors' knowledge, this paper is so far the only prospective randomized study showing that IMSI is significantly more beneficial than ICSI on all patients with severe oligoasthenoteratozoospermia, regardless of the number of previous IVF failures. Those cases with two or more failed attempts, despite their poor reproductive prognosis, seem to benefit with statistically significant doubling of the pregnancy

rate ($P = 0.017$) and remarkable halving of the miscarriage rate. It is the opinion of the authors that in the near future, after adequate standardization of the method in order to gain insight into the selection criteria and overcome some of the above practical difficulties, IMSI could be recommended as a routine IVF technique to solve complicated male infertility cases from their first attempt.

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