Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection

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Objective: To verify whether microinjection into retrieved oocytes of motile spermatozoa with morphologically normal nuclei, strictly defined by high power light microscopy ($\times$ >6000), improves the IVF/intracytoplasmic sperm injection (ICSI) pregnancy rate in couples with repeated ICSI failures.

Design: Comparative prospective study testing routine IVF/ICSI outcome parameters against those of modified ICSI based on morphological selection of spermatozoa with normal nuclei.

Setting: Male factor fertility laboratory and IVF center.

Patient(s): Sixty-two couples, with at least two previous consequent pregnancy failed ICSI cycles, underwent a single ICSI trial preceded by morphological selection of spermatozoa with normal nuclei. Fifty of these couples were matched with couples who underwent a routine ICSI procedure at the same IVF center and exhibited the same number of previous ICSI failures.

Intervention(s): Standard ICSI and modified ICSI.

Main Outcome Measure(s): ICSI pregnancy rate.

Result(s): The matching study revealed that pregnancy rate after modified ICSI was significantly higher than that of the routine ICSI procedure (66.0% vs. 30.0%).

Conclusion(s): Microinjection into retrieved oocytes of selected spermatozoa with strictly defined morphologically normal nuclei improves significantly the incidence of pregnancy in couples with previous ICSI failures. (Fertil Steril® 2003;80:1413–9. ©2003 by American Society for Reproductive Medicine.)

Key Words: IVF/ICSI, pregnancy rate, sperm morphological selection, sperm nucleus, male fertility

Intracytoplasmic sperm injection (ICSI) is, currently, the most efficient variant of micromanipulation-assisted fertilization, whereby one spermatozoon is selected, aspirated into a microinjection needle, and injected into the oocyte cytoplasm. Several related steps are included in this assisted reproduction technique: selection of patients; ovarian stimulation; retrieval and handling of oocyte; evaluation and preparation of spermatozoa; the micromanipulation itself; pronucleus formation after ICSI; embryo development in vitro and replacement into the uterus. Although, in cases of male factor infertility, ICSI has almost become a routine laboratory service, the resulting pregnancy rates are only 30%–45% (1–6). There-fore, more efficient variations of the ICSI technique are still desirable (7, 8).

The present study refers to the step of evaluation and preparation of spermatozoa. A new method of unstained, real-time, high magnification motile sperm organellar morphology examination (MSOME) was developed in our laboratory (9). Application of this method to patients undergoing conventional IVF/ICSI treatment demonstrated that fine morphological integrity of the human sperm nuclei is an important parameter associated with ICSI pregnancy rate. Because MSOME is an unstained cytological technique, its incorporation, together with a micromanipulation system, enables retrieval of a single spermatozoon with

defined morphology. This option warrants the initiation of a clinical study involving exclusive selection of spermatozoa free of any nuclear morphological malformations to be injected into retrieved oocytes.

The aim of this prospective study was to verify that a single microinjection of selected motile spermatozoa with strictly defined morphologically normal nuclei into the cytoplasm of retrieved oocytes improves the IVF/ICSI pregnancy rate in couples with repeated IVF/ICSI failures. We named this modified IVF procedure: intracytoplasmic morphologically selected sperm injection (IMSI).

MATERIALS AND METHODS

Patients

The study included couples directed for IVF/ICSI treatment to the IVF unit at Herzliya Medical Center, Israel, from December 2000 to June 2002. The indication for ICSI was male factor infertility, defined according to WHO criteria (10) for sperm density, motility, and morphology, which has previously been tested in other laboratories. If at least one of these three parameters was abnormal the couple was considered for ICSI treatment. Those couples who fitted the following selection criteria: at least two previous consequent pregnancy failed routine ICSI cycles with one performed within 6 months before the IMSI procedure at the same IVF center; female partner younger than 37 years; and more than three retrieved M-II ova in the last ICSI cycle; were proposed to undergo a single IMSI trial, which was approved by the Meir Hospital ethical committee. Eighty couples agreed to undergo this modified micromanipulation method and written, informed consent was obtained from each couple.

During sperm selection before IMSI, we were unable to find any spermatozoa with morphologically normal nuclei in 18 of the recruited couples, which made them unsuitable for the new treatment. These couples were excluded from the study and directed for a routine ICSI trial (see Results section).

Thus, finally, only 62 IMSI couples were included in this study, 50 of them were matched with couples who continued in routine ICSI treatment (current ICSI). The matching criterion was the number of previous failed ICSI trials. The search for a matching ICSI couple continued for up to 1 month after IMSI treatment. No matches were found for any of the IMSI couples who had previously undergone more than eight failed routine ICSI cycles (n = 12, 9.1 ± 1.2 cycles in average, range 9–13 cycles; Table 1). These couples were defined as unmatched IMSI cases.

The matched IMSI and ICSI couples comprised the experimental and the control groups, respectively. The average number of previous failed routine ICSI treatments in these groups was 4.1 ± 1.9 cycles (range 2–8 cycles). The last ICSI attempt before the IMSI procedure was designated as the previous ICSI procedure for the experimental group.

The ICSI trial preceding the current ICSI procedure in the control group was designated as the previous ICSI procedure for this group. In either of the two groups paired t tests revealed no significant difference between the single previous ICSI procedure and the pooled ICSI cycles for number of retrieved and injected ova, fertilization rate, percent of top embryos, or number of transferred embryos.

Unpaired Student's t tests revealed no statistical difference between the experimental and the control groups regarding average age of the female and male partners or time of pregnancy expectation (Table 1). Similarly, no statistical difference was found concerning the average values of sperm density or percent of motility or morphology, defined according to WHO criteria (10) (Table 1).

Comparison between the experimental group and the unmatched IMSI cases, using unpaired Student's t tests, revealed that the latter group exhibited significantly higher numbers of previous ICSI failures and years of pregnancy expectation ($t = -9.8$ and $t = -3.4$, respectively, $P \leq .01$; Table 1). No significant difference between these two groups was found in basic sperm parameters, defined according to WHO criteria (10) (Table 1).

IMSI Procedure

Routine Preparation of Motile Sperm Fraction

Only freshly ejaculated semen was used in this study. All technicians were blinded regarding each patient's prior medical history. The routine morphological selection of motile spermatozoa was performed on the basis of a two-layer Sil-Select density gradient system, which consists of 1-mL upper (low density) and 1-mL lower (high density) layers of

silane-coated colloidal silica particles suspended in HEPES-buffered Earle's balanced salt solution (EBSS; Ghent, Belgium). Up to 1 mL of postejaculated liquefied semen was gently placed onto the gradient and centrifuged at $375 \times g$ for 15 minutes at 25°C. The sperm cell pellet was suspended by adding 3 mL of SPERM medium (Medi-Cult, Jyllinge, Denmark) and then recentrifuged for 10 minutes. The supernatant was removed and replaced with SPERM medium to bring the final concentration of motile sperm cells to about 4×10^6 spermatozoa per milliliter. In 12 severe oligozoospermic cases with sperm density below 1×10^6 spermatozoa per ejaculate, liquefied semen was placed onto 1 mL of the low density layer only, centrifuged or washed as previously, and the final sperm cell pellet was suspended in 0.1–0.2 mL of SPERM medium. The sperm cells suspension was used for further MSOME preparation.

Sperm Preparation for Retrieval Based on MSOME

An aliquot of 2–4 μL of the sperm cell suspension, containing a few thousand spermatozoa, was transferred to a 4-μL microdroplet of SPERM medium containing 0–8% polyvinyl pyrrolidone (PVP) solution (PVP medium 10890001, Medi-Cult). This microdroplet was placed in a sterile, glass-bottomed dish (BioSoft International, GWSt-1000, Amsterdam, The Netherlands) under sterile paraffin oil (OVOIL-100; Vitrolife, Goteborg, Sweden). The temperature of the sperm sample and the PVP concentration were coordinated with the intensity of the sperm motility (9). The sperm cells, suspended in the microdroplet, were used for individual retrieval by MSOME. For this purpose, the sterile glass bottom dish containing the microdroplet (observation droplet) was placed on a microscopic stage over the top of an Uplan Apo $\times$100 oil/1.35 objective lens previously covered by a droplet of immersion oil. In this way the motile sperm cells, suspended in the observation droplet, could be examined at high magnification by the inverted microscope (Olympus IX 70, Tokyo, Japan) equipped with Nomarski differential interference contrast optics. The total calculated magnification was $\times$6,600.

MSOME Criteria for Spermatozoa Suitable for IMSI

The MSOME criteria for the morphological normalcy of the sperm nucleus were defined according to the arbitrary descriptive approach adopted in our previous ultramorphological studies, which compared spermatozoa of fertile and infertile males (11, 12). Based on sperm observation by scanning electron microscopy (providing detailed information on the sperm head external structure) and transmission electron microscopy (providing two-dimensional internal information on the karyoplasms) we came to the conclusion that the MSOME criteria for normally shaped nuclei (based on Nomarski differential interference contrast optic, supplying both external and internal information) are smooth, symmetric, and oval configuration (an extrusion or invagination of the nuclear mass was defined as a regional nuclear shape

malformation) and homogeneity of the nuclear chromatin mass containing no more than one vacuole, which occupies less than 4% of the nuclear area. The average length and width limits, estimated by MSOME in 100 spermatozoa with a normally looking nucleus, were found to be 4.75 $\pm$ 0.28 μm and 3.28 $\pm$ 0.20 μm, respectively.

Sperm Retrieval for IMSI

Spermatozoa with morphologically normal nuclei (16.5 $\pm$ 5.0 sperm cells per couple in average, range 3–28 spermatozoa) were retrieved from the observation droplet and placed into a recipient selection droplet, containing 4 μL of SPERM medium in the same WillCo dish. This procedure was performed using the Eppendorf Micromanipulation System 5177000.010 and Celltram oil 5176000.02 (Eppendorf-Nethelei-Hinz GmbH, D-2000, Hamburg, Germany), which is equipped with a sterilized, nonangulated, glass microcapillary with a 12-μm diameter tip. The glass microcapillary was inserted from above into the observation droplet from the condenser lens direction.

Preparation for IVF

The preparation for the IVF/IMSI procedure was conducted according to the method described by Van Steirteghem et al. (13). Commencing on day 21 of the previous cycle or on days 1–3 of the current cycle, the female partner received GnRH agonist depot or daily leuprorelin (Decapeptyl) 3.75 depot or triptorelin (Decapeptyl) 0.1 mg or intranasal nafarelin (Synarel) 400–800 μg. A serum E_2 level of <50 pg/mL and absence of ovarian cyst 2 cm on ultrasound scan confirmed pituitary down-regulation. When pituitary desensitization was achieved, ovarian stimulation was started with human recombinant stimulating hormone rFSH (Follitrophin alpha, marketed as Gonal-F by Serono, Geneva, Switzerland). Final oocyte maturation was induced with 10,000 IU hCG when at least two follicles with a mean diameter of >18 mm were observed. Oocyte retrieval was performed by transvaginal ultrasound needle-guided aspiration at 34 hours after hCG injection.

Microinjection

The transferred, retrieved cumulus-free ova were placed into drops of SPERM medium, prepared in the same glass dish with the recipient droplet. The latter contained the sperm cells morphologically selected for IMSI. Microinjection of the selected spermatozoa into the oocytes was conducted in SPERM medium. Each inseminated oocyte was immediately transferred to a four-well dish (Nunc), incubated in 0.5 mL of IVF or ISM 1 medium (Medi-Cult, New York, NY), covered with 0.5 mL of mineral oil (Medi-Cult) in 37°C with an atmosphere of 5% CO_2.

Fertilization was recorded if two pronuclei could be detected after 12–18 hours after microinjection. Fertilization rate was calculated as the percentage of fertilized ova resulting from the number of injected ova. Twenty-four hours after

Comparison between the different study groups in IVF outcome parameters.

IVF outcome parameters	Control (n = 50)		Experimental (n = 50)		Unmatched IMSI[d] (n = 12)	
	Previous procedure (ICSI)	Current procedure (ICSI)	Previous procedure (ICSI)	Current procedure (IMSI)	Previous procedure (ICSI)	Current procedure (IMSI)
Retrieved ova	13.3 ± 5.2	13.2 ± 5.9	13.4 ± 5.5	13.6 ± 5.8	12.2 ± 6.5	14.0 ± 6.8
Injected ova	10.3 ± 4.7	10.2 ± 5.5	10.1 ± 4.6	10.6 ± 4.4	9.4 ± 4.6	11.2 ± 4.7
Fertilization rate (%)	63.7 ± 18.9	65.5 ± 21.5	63.1 ± 25.3	64.5 ± 17.5	70.1 ± 12.3	70.0 ± 14.5
Percentage of top embryos (%)	31.1 ± 16.4	31.0 ± 19.5	31.7 ± 21.6	45.2 ± 28.2[b,c]	31.7 ± 16.2	40.8 ± 30.7
Transferred embryos	3.6 ± 1.1	3.5 ± 1.2	3.6 ± 1.2	3.8 ± 1.1	3.7 ± 1.5	3.9 ± 1.1
Implantation rate (%)[a]	—	9.5 ± 15.3	—	27.9 ± 26.4[c]	—	22.4 ± 23.0
	Nr	(%)	Nr	(%)	Nr	(%)
Pregnancy occurrence[a]	—	15	30	—	33	66[c]	—	6	50
Missed abortion[a]	—	5	33	—	3	9[c]	—	0	0

Note: Values are means ± SD.
[a] Not included in repeated measurements MANOVA analysis.
[b] Significantly different from the previous ICSI procedure ($P \leq .01$).
[c] Significantly different from the current ICSI procedure ($P \leq .01$).
[d] The unmatched IMSI group was compared with the experimental group only.

Bartoov. Sperm morphological selection and ICSI. Fertil Steril 2003.

sperm injection the oocytes with two pronuclei were replaced in fresh droplets of IVF or ISM 1 in a new four-well dish. Forty-eight hours after insemination the embryos were replaced in fresh droplets of ISM 2 in a new four-well dish. Embryos with less than 10% fragmentation and four blastomers on day 2, or eight blastomers on day 3, were defined as top embryos. The percentage of top embryos was defined as the number of top embryos obtained from the total number of cleaved embryos. Embryo transfer was usually performed on day 3. However, when day 3 fell on Saturdays or Israeli holidays, embryo transfer was performed on day 2. The implantation rate was calculated as the percentage of gestational sacs, observed by ultrasound, resulting from the total number of transferred embryos. Clinical pregnancy was confirmed at 6 weeks by ultrasound examination.

Conventional IVF/ICSI Procedure

Indications for ICSI were similar to those described for IMSI. Sperm selection for microinjection was performed at a magnification of ×200–×400. The embryologist excluded from microinjection into the ovum spermatozoa with severe head-shape defects clearly seen under this magnification (pin, amorphous, tapered, round, and multinucleated head). No sperm selection based on MSOME was performed before conventional ICSI.

Statistical Analysis

All statistical analyses were performed using SPSS for Windows Version 10.0 (SPSS Inc., Chicago, IL). Data were presented as means ± SD for continuous variables and as percentages for discrete variables. The couples included in this study received one of the two different kinds of current treatment: IVF/IMSI or IVF/ICS. The variable current treatment divided the subjects into the experimental and control groups and was used for statistical analysis as a between-subject factor. The following IVF outcome parameters—number of retrieved and injected ova, fertilization rate, percent of top embryos, and number of transferred embryos—were repeatedly measured at two different points of evaluation: after the previous and after the current IVF attempts. Therefore, the variable time of evaluation was defined as within-subject factor. Changes in these IVF outcome parameters in the matched study groups were analyzed by repeated measurement multivariate analysis of variance (MANOVA) with current treatment, as between factor, and time of evaluation, as within factor. Comparisons between the current IVF procedures in continuous and discrete variables were performed using unpaired Student's t tests and χ^2 tests, respectively. The latter statistical methods were used to compare the experimental study group and the unmatched IMSI cases. Pearson's correlation analysis was used to identify any relationship between pregnancy occurrence and demographic data or basic semen variables.

RESULTS

The average values of the IMSI outcome parameters in the experimental study group are demonstrated in Table 2. The total numbers of retrieved and injected ova were 667 and 519, respectively, and the total number of transferred embryos was 188. Notably, although all 50 couples failed to conceive after previous IVF/ICSI attempts (4.1 ± 1.9 cycles

per couple), the current IMSI treatment resulted in a pregnancy rate of 66.0% (n = 33). The distribution of singleton, twin, triplet, and quadruplet pregnancies was 17 (51.6%), 14 (42.4%), 1 (3.0%), and 1 (3.0%), respectively. In the case of quadruplet pregnancy, one embryo was diluted. In 18 couples, natural delivery has already taken place. Of 31 infants delivered so far, 30 are normal and healthy. One died during delivery due to vasa previa. A further 12 pregnancies are ongoing. In one of these cases, one heterotopic (intrauterine + extrauterine pregnancy) occurred. Three pregnancies (9.0%) terminated in a first trimester missed abortion.

Comparison between the experimental and control groups in number of retrieved and injected ova, fertilization rate, percent of top embryos, and number of transferred embryos, using repeated multivariate analysis of variance (MANOVA) measurements, revealed a significant interaction between the current treatment (IMSI/ICSI) and time of evaluation (previous procedure/current procedure) (Hotelling's F = 2.7, $P \leq .02$), based on percentage of top embryos only. The IMSI attempt produced a significantly higher value of this outcome parameter, compared to the current ICSI treatment (F = 6.5, $P \leq .01$; Table 2).

As according to the selection criteria, no pregnancy occurred in the previous IVF procedures, we did not include the pregnancy outcome parameters (implantation, pregnancy, and abortion rates) in the repeated MANOVA measurements. Comparison between the current IMSI and ICSI treatments concerning these three outcome parameters demonstrated that implantation and pregnancy rates after IMSI were significantly higher, and the abortion rate was significantly lower, compared to the current ICSI trial (F = 18.0, $P \leq .01$; $\chi^2 = 4.4$, $P \leq .01$; and $\chi^2 = 4.4$, $P \leq .05$; Table 2).

The previous ICSI outcome parameters did not differ significantly between the experimental and control groups (Table 2).

No significant correlation was found between demographic data and basic semen quality with pregnancy outcome after any of the current or previous micromanipulation procedures.

Comparison between the experimental study group and the unmatched IMSI cases demonstrated no statistical difference between the two groups in any of the IMSI outcome parameters (Table 2).

Of the 18 eliminated patients who underwent a current routine ICSI trial, as they did not exhibit any sperm cells suitable for IMSI, only one achieved pregnancy after the conventional procedure. This pregnancy resulted in an early natural abortion.

DISCUSSION

In a recent Letter to the Editor (14), we reported a high pregnancy rate of 67% after a single IMSI trial of 24 couples with at least five previous failed consecutive routine IVF/ICSI cycles. To validate this significant result, a matching study comparing the new IMSI procedure with the conventional IVF/ICSI was necessary. To eliminate the effect of the number of previous ICSI failures on the probability of achieving pregnancy after micromanipulation (1, 4–6) we used the number of previous failed ICSI trials as a matching criterion.

The present study confirms the advantage of the modified IMSI trial over the conventional IVF/ICSI procedure. It is important to emphasize that both micromanipulation procedures were performed under identical conditions (patient selection criteria, IVF center, staff, and equipment) with one exception—sperm selection based on high power microscopy (×6,000) observation—performed exclusively for the IMSI procedure. Thus, the significantly higher pregnancy rate achieved after the latter treatment (66% vs. 30%) could be attributed to this one difference.

As has been recently reported, the conventional IVF/ICSI pregnancy rates range between 30% and 45% (1–6). It should be noted that the latter reports also included couples with either none or one previous ICSI failure, whereas the participants of this study underwent at least two consecutive routine cycles of ICSI (4.1 ± 1.9 micromanipulation failures on average). According to Stolwijk et al. (6), the statistical probability of the latter couples to conceive is quite low. Nevertheless, the pregnancy results obtained in the control group (30%) are comparable with those reported by other investigators. These results may probably be explained by the fact that mild male factor infertility cases, based on only one abnormal basic sperm parameter, were also included in this study. However, the 66% pregnancy rate in the experimental group appears to be much higher than expected.

The single IMSI attempt pregnancy rate is similar to the cumulative pregnancy rates calculated by Olivius et al. (5) and Stolwijk et al. (6) after three and five consecutive ICSI trials (66.3% and 54.5%, respectively). It should be emphasized that even the 12 unmatched IMSI cases, with more than eight failed routine ICSI attempts (9.1 ± 1.2 ICSI cycles in average), achieved a 50% pregnancy rate after one IMSI trial.

From these results one may assume that in the IVF/ICSI procedure, the survival of the embryo in the uterus is associated with the fine morphological state of the sperm nucleus. Therefore, IMSI, based on exclusive selection of spermatozoa free of any subtle nuclear morphological malformation, leads to a higher pregnancy outcome.

New results reported by De Vos et al. (15), which found that significantly lower pregnancy and implantation rates were obtained after transfer of embryos resulting from morphologically abnormal sperm cells, are in full agreement with this conclusion.

This conclusion is also supported by two findings in this study: that early spontaneous abortion rate, obtained after IMSI, was significantly lower than that of the current matched ICSI trial and also lower than the routine ICSI abortion rate recently reported by other investigators (16, 17); and that of all analyzed IMSI outcome parameters, only a percentage of top embryos showed a significant relation with treatment.

However, the improvement in embryo quality (33%) cannot, by itself, account (if at all) for the sharp increase in pregnancy and implantation rates (200% and 300%, respectively) after IMSI. To explain this phenomenon we accept the assumption of De Vos at al. (15), that the underlying reason for the relationship between individual sperm morphology and improved pregnancy and implantation rates is probably based on hidden defects at the chromosomal level of the embryo contributed by the sperm cell.

It has recently been reported by Larson et al. (18) that sperm chromatin integrity, assessed by the sperm chromatin structure assay (measuring susceptibility to DNA denaturation), exhibits no correlation with the fertilization rate after ICSI. However, chromatin abnormalities appear to influence later embryonic development, demonstrated by the absence of clinical pregnancy in patients with sperm DNA denaturation exceeding a threshold of 27% of spermatozoa in their sample. Based on this report one may speculate that subnormal sperm chromatin integrity seems to be a potential cause for ICSI-resistant couples.

The fact that 62% of these ICSI-resistant couples have conceived after a single IMSI trial without a significant change in the fertilization rate emphasizes a possible association between sperm chromatin abnormalities, assessed by sperm chromatin structure assay, with the morphological state of the sperm nucleus, as defined by MSOME. This indication warrants the initiation of a clinical study involving IMSI treatment in couples with male partners exhibiting high levels of acid-induced DNA denaturated spermatozoa.

The abnormal morphological state of the sperm nucleus is probably not the only obstacle to achieve pregnancy after conventional ICSI. For example, patients with subnormal sperm hypoosmotic swelling test scores are known to exhibit very low implantation of embryo after conventional IVF, despite normal fertilization rates. As reported by J. H. Check et al. (19) and M. L. Check et al. (20), this phenomenon seems to be related to toxic factors attached to the sperm that are transferred to zona pellucida and may be corrected to some degree using conventional ICSI. We do not expect any advantage of the IMSI trial over the conventional IVF/ICSI procedure in cases of low hypoosmotic swelling test scores, as no morphological factors seem to be involved in these IVF failures.

In addition, there is an accumulation of data correlating oocyte morphology with pregnancy rate after ICSI (21, 22).

As is the case in the low hypoosmotic swelling test score condition, IMSI does not seem to be an efficient treatment for couples with abnormal oocyte quality. These assumptions should be further investigated in patients with successful sperm selection.

As in other recently reported ICSI studies (23, 24), we did not find any relationship between the basic routine sperm characteristics and IMSI outcome parameters. This fact is not surprising, as the few spermatozoa with no nuclear abnormality, selected before microinjection, do not necessarily represent the total sperm population in the ejaculate.

The present study demonstrated that in 22% of the couples, fitting the selection criteria, no spermatozoa with morphologically normal nuclei were available for injection into retrieved ova. Accordingly, MSOME may serve as a diagnostic tool to enable the identification of which sperm samples may or may not be suitable for IMSI.

We conclude that the fine morphological state of the sperm nucleus is an important factor in achieving pregnancy after ICSI. Developed in our laboratory, MSOME may serve as a practical tool to isolate spermatozoa with strictly defined morphologically normal nuclei for microinjection into the ovum. The combination of these two procedures should increase the efficiency of assisted reproduction technology (ART). To validate these conclusions, it is strongly recommended that a double-blind multicenter study be performed subsequent to this present investigation.

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