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Intracytoplasmic sperm injection: a novel treatment for all forms of male factor infertility

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Objective: To demonstrate the effectiveness of intracytoplasmic sperm injection to treat male factor infertility and to report on the achievement of fertilization and pregnancy compared with standard in vitro insemination.

Design: Controlled clinical study.

Setting: Couples suffering from male factor infertility treated in an academic research environment.

Patients: Two hundred twenty-seven couples in whom the male partners were presumed to be the cause of repeated failed attempts at IVF or whose semen parameters were unacceptable for conventional IVF.

Interventions: Oocytes for this study were harvested after superovulation with GnRH agonist and gonadotropins. After removing the cumulus cells, a single spermatozoon was injected directly into the cytoplasm of metaphase II oocytes, and the outcome was analyzed in terms of semen parameters and origin of semen sample.

Main Outcome Measures: Fertilization and pregnancy rates (PRs) in relation to sperm parameters and origin of semen sample.

Results: Two hundred twenty-seven couples were treated by intracytoplasmic sperm injection resulting in an ongoing pregnancy rate of 37.0% per retrieval (84/227). There were 47 singleton pregnancies (5 of which were vanishing twin pregnancies), 30 twin gestations, 6 triplet pregnancies, and 1 quadruplet pregnancy. The concentration of the total motile spermatozoa present in the ejaculate as well as the origin of the samples influenced the fertilization rate but not the pregnancy outcome.

Conclusions: Intracytoplasmic sperm injection can be used successfully to treat couples who have failed IVF or who have too few spermatozoa for conventional methods of in vitro insemination. Sperm parameters do not clearly affect the outcome of this technique.

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Key Words: Assisted fertilization, human sperm injection, micromanipulation, intracytoplasmic injection, human oocytes

In vitro fertilization by insemination has been used to treat couples with male factor infertility for over a decade with relatively disappointing results. Fertilization failure is especially common when patients have grossly abnormal semen parameters or when insufficient spermatozoa are retrieved. This has led to the application of gamete micromanipulation. Opening the zona pellucida by either chemical, mechanical, or photoablation has enhanced fertilization rates, bypassing obstacles to sperm penetration where standard procedures of in vitro insemination have failed (1–3). The injection of spermatozoa under the zona pellucida (ZP) has demonstrated consistent and reproducible results achieving a fertilization rate of approximately 20% (4). Some of the limiting factors, however, are the requirements for many functional spermatozoa with good progressive

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motility, the presence of a normal acrosome, acrosomal content, and dynamic of the acrosomal loss. Furthermore, these techniques together with a poor fertilization rate presented a higher incidence of multiple sperm penetration when the number of inserted spermatozoa was increased (5).

Manipulation of the human fertilization process by deposition of a single sperm cell into the cytoplasm of the oocyte was preceded by experimental work in hamsters and mice using sperm nuclei and membrane relaxants (6, 7). The capability of injecting sperm directly into the cytoplasm of a human oocyte (bypassing the ZP and the oolemma), achieving decondensation and male pronucleus formation, has offered new possibilities for male gametes with poor motility and abnormal or absent acrosomes (8, 9). It was demonstrated that this approach is superior to subzonal insemination, after the first four human pregnancies by intracytoplasmic sperm injection were reported by Palermo et al. (10) in 1992 (11, 12). Since then, there has been a tendency to abandon the subzonal approach as the intracytoplasmic sperm injection technique has become increasingly popular because of the higher fertilization rates.

This study reports on 227 consecutive cycles of assisted fertilization by intracytoplasmic sperm injection in 227 couples and deals with three relevant issues. First, what are the cutoff limits, if any, between the frequency of motile spermatozoa in the semen sample (regardless of origin) and, second, what is the efficiency of intracytoplasmic sperm injection in terms of normal fertilization, cleavage, and implantation? The third and most important issue concerns the differences between intracytoplasmic sperm injection and IVF and the predictability of success once the sperm is inserted directly into the ooplasm.

MATERIALS AND METHODS

Patients

From September 29, 1993 through March 14, 1994, assisted fertilization was performed by intracytoplasmic sperm injection in 227 couples with long-standing infertility. The mean age of the female partner was 35.0 years (range 26 to 43 years). The intracytoplasmic sperm injection technique was reviewed and approved by the Committee on Human Rights of The New York Hospital-Cornell Medical Center. The couples selected to participate in the assisted fertilization program were patients with male factor or idiopathic infertility. The idiopathic indication included couples who were unable to achieve fertilization in vitro in a previous attempt (n = 117) and others with severely compromised sperm parameters (i.e., severe oligospermia and/or asthenospermia).

The couples were counseled about intracytoplasmic sperm injection as a new assisted reproduction technique and were informed of the many unknown aspects of this new treatment. The male partner was asked to produce a preliminary sperm sample several weeks before the planned ovum pick-up, upon which a routine semen analysis was performed. The male partner was informed that at the time of the actual IVF attempt he might be asked to produce additional semen samples.

Semen Collection

When possible, semen samples were collected by masturbation after ≥3 days of abstinence and were allowed to liquefy for at least 20 minutes at 37°C before analysis. When the semen presented with a high viscosity, the patient was asked to ejaculate into a jar containing 2 to 3 mL of 20% HEPES-buffered human tubal fluid (HTF-HEPES) containing 200 to 500 IU of chymotrypsin (Sigma Chemical Co., St. Louis, MO), depending on the severity of the viscosity. Reduction of viscosity generally followed within 3 to 5 minutes (13).

Electroejaculation

Electroejaculation was performed by using the rectal probe electrode developed by Seager and reported by Bennett et al. (14). Urinary alkalization before each stimulation procedure involved a 2-day regimen of an oral sodium bicarbonate treatment. Nifedipine (10 mg) was administered 30 minutes before each stimulation procedure to those patients at risk for autonomous dysreflexia. Electroejaculation began with catheterization of the bladder to drain the urine, followed by intravesical instillation of 30 mL of HTF-HEPES medium supplemented with bovine serum albumin (BSA). The antegrade portion of the ejaculate was obtained by manual expression of seminal fluid along the perineal and penile urethra whereas the retrograde portion of the ejaculate was recovered by recatheterization and draining the contents of the bladder (15).

Epididymal Sperm Sampling

To eliminate any possible detrimental effects of antisperm antibodies, all patients were treated with oral prednisone (20 mg/d for 7 d/mo) unless its administration specifically was contraindicated. Surgical exploration of the testis was performed through a midline scrotal incision. The testis was delivered and the tunica vaginalis was opened immediately if

no vas deferens was found. Variable volumes of fluid (1 μL to 100 μL) from the epididymal lumen were collected on the initial puncture by using a micropipette with a tip width of 250 to 350 μm; the epididymis and testis were gently compressed to facilitate sperm collection. When the flow of fluid ceased, the micropipette was removed and the fluid was placed in 500 μL of HTF medium. Additional proximal punctures were performed progressively until it was evident empirically that optimal sperm quality had been obtained from the epididymis. It is important to note that spermatozoa are highly concentrated in the epididymal fluid and concentrations $> 1 \times 10^6$/μL are found frequently. Thus, only microliter quantities of fluid are needed (16).

Semen Analysis, Classification, and Selection

Semen concentration and motility were assessed in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) according to criteria described previously (17). Semen morphology was then assessed by spreading 5 mL of semen or sperm suspension on prestained slides (Blustan slides; Irvine Scientific, Santa Ana, CA). The morphology parameters were evaluated and classified according to the strict criteria described by Kruger et al. (18). Semen parameters were considered compromised when sperm density was $<20 \times 10^6$/mL, progressive motility was <40%, or when <5% of the spermatozoa had a normal morphology.

The sperm sample was washed by centrifugation at 500 × *g* for 5 minutes in HTF medium supplemented with 30 mg/mL BSA fraction V (A-9647; Sigma Chemical Co.). Semen samples with $<5 \times 10^6$/mL spermatozoa or <20% motile spermatozoa were washed in HTF medium by a single centrifugation at 1,800 × *g* for 5 minutes. The resuspended pellet was layered on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) on three layers (90%, 70%, and 50%) and centrifuged at 300 × *g* for 20 minutes. A Percoll gradient in two layers was used (95% and 47.5%) when samples had a sperm density $< 5 \times 10^6$/mL and <20% motile spermatozoa. The Percoll fraction containing the spermatozoa was washed twice by adding 4 mL of HTF medium and centrifuged at 1,800 × *g* for 5 minutes to remove the silica gel particles. For spermatozoa with poor kinetic characteristics, the sperm suspension was exposed to a 3 mM solution of pentoxifylline and deoxyadenosine and was washed again. The concentration of the assessed sperm suspension was adjusted to 1 to 1.5×10^6/mL, when necessary, by the addition of HTF medium and subsequently incubated at 37°C in a gas atmosphere of 5% CO_2 in air.

Collection and Preparation of Oocytes

Female partners were down-regulated with 1 mg SC luteal GnRH agonist (GnRH-a) daily and subsequent ovarian superovulation was carried out by administering a combination of hMG (Pergonal; Serono, Waltham, MA) (n = 137 couples) and/or pure FSH (Metrodin; Serono) (n = 15 couples). Down-regulation by 0.5 mg SC low-dose GnRH-a daily was administered in 67 couples. In some cases direct ovarian superovulation, without GnRH-a, was performed by administering FSH (n = 4 couples), hMG (n = 2 couples), or clomiphene citrate plus hMG (n = 2 couples). Human chorionic gonadotropin was administered when criteria for oocyte maturity were met and oocyte retrieval by vaginal ultrasound (US)-guided puncture was performed 35 hours after hCG administration (19). Under the inverted microscope at 40× or 100× magnification the cumulus-corona cell complexes were scored as mature (A), slightly immature (B), completely immature (C), or slightly hypermature (PM). Thereafter, the oocytes were further incubated for ≥4 hours. Immediately before micromanipulation, the cumulus-corona cells initially were removed by exposure to HTF-HEPES-buffered medium (21 mM HEPES concentration) containing 80 IU/mL of hyaluronidase (type VIII; Sigma Chemical Co.). The HTF medium was prepared in our laboratory according to Quinn et al. (20).

To enhance the enzymatic removal of the cumulus and corona cells, the oocytes were aspirated in and out of a hand-drawn Pasteur pipette with an approximate inner diameter of 200 μm. An attempt to remove completely the adhering corona radiata was necessary because residual corona cells can limit observation of the oocyte and block the holding and/or injecting pipettes. Each oocyte was washed twice in HTF medium supplemented with 10% patient serum and then examined under the inverted microscope at 200× magnification to assess integrity and maturation stage. The latter was performed by observing the presence of the germinal vesicle, germinal vesicle breakdown, and the extruded first polar body (metaphase II stage). Intracytoplasmic sperm injection was executed on all oocytes that had reached the metaphase II stage.

Setting for the Microinjection

The pipettes were made from borosilicate glass capillary tubes (Drummond Scientific, Broomall, PA) with a 0.97 mm external diameter, 0.69 mm internal diameter, and 78 mm length. Cleaning of the capillary tubes involved the following steps: [1] soaking overnight in MilliQ water (Millipore Corporation, Bedford, MA); [2] sonicating; [3] rinsing ≥30 times in MilliQ water before drying in a hot-air oven; and

[4] heat sterilization. The glass pipettes were made by drawing the thin-walled glass capillary tubes using a horizontal microelectrode puller (model 753; Campden Instruments Ltd., Loughborough, United Kingdom). The holding pipette was cut and fire-polished on a microforge (Narishige Co. Ltd., Tokyo, Japan) to obtain a final outer diameter of 60 mm and an inner diameter of 20 μm. To prepare the injection pipette, the pulled capillary was opened and sharpened on a grinder (Narishige Co. Ltd.); the bevel angle was 30°, the outer diameter was approximately 7 mm, and the inner diameter was approximately 5 mm. On the microforge a spike was made on the injection pipette and both pipettes were bent to an angle of approximately 40° at 1 mm from the edge to be able to perform the injection procedure with the tip of the tools horizontally positioned in a plastic Petri dish (model 1006, Falcon, Becton & Dickinson, Lincoln Park, NJ).

Immediately before the injection procedure, 1 μL of sperm suspension was diluted with 4 μL of a 10% polyvinyl pyrrolidone solution (PVP-K 90, molecular weight 360,000; ICN Biochemicals, Cleveland, OH) in HTF-HEPES medium placed in the middle of the petri dish. The PVP solution was made using the reagent direct from the manufacturer without further purification. The osmolarity of the PVP solution was corrected to approximately 290 mOsmol. The use of this viscous solution was necessary to decelerate the aspiration and to prevent the sperm cells from sticking to the injection pipette during the procedure. When <500,000 spermatozoa were present in the ejaculate, the sperm suspension often was concentrated in approximately 5 μL and transferred directly into the injection dish. The spermatozoon was aspirated from the concentrated 5 μL sperm suspension and transferred into the central drop containing PVP solution, to remove debris and facilitate aspiration control.

Each oocyte then was placed in a droplet of 5 μL medium surrounding the central drop containing the sperm suspension-PVP. HTF-HEPES medium supplemented with 5 mg/mL BSA (A-3156; Sigma Chemical Co.) was used in the injection dish. The droplets were covered with lightweight paraffin oil (BDH Limited, Poole, United Kingdom). Up to four oocytes were placed in the injection dish to avoid exposure to the HEPES-buffered medium longer than 10 minutes. The droplets of each dish were used only once to avoid dilution of the buffered medium during the transport of the oocyte.

The procedure was carried out on the heated stage (Easteach Laboratory, Centereach, NY) of a Nikon Diaphot inverted microscope at 400× magnification using Hoffman Modulation Contrast (Modulation Optics, Greenvale, NY) optics. This microscope was equipped with two motor-driven coarse control manipulators and two hydraulic micromanipulators (MM-188 and MO-109; Narishige Co., Ltd.). The micropipettes were fitted to a tool holder controlled by two IM-6 microinjectors (Narishige Co., Ltd.).

Selection of the Spermatozoon

When the 1 μL of sperm suspension was added to the drop containing PVP, motile spermatozoa progressed into the viscous medium where debris, other cells, bacteria, and immotile spermatozoa floated in the PVP at its interface with the paraffin oil. In addition to decelerating the spermatozoa, this viscous environment highlights their tridimensional motion patterns, which allows for careful observation of the cell, thereby facilitating its aspiration into the pipette.

Preferential choice for aspiration went to spermatozoa that tended to stick to the bottom of the petri dish by their heads and that usually became quickly immotile after displaying simple vibration patterns for a short period. Generally, these spermatozoa are found lying on the bottom of the dish at the edge of the droplet, completely immotile (although viable); their immotility being obviously recent. For intracytoplasmic sperm injection, these spermatozoa offer the highest chances of achieving fertilization once correctly injected.

Nonetheless, some spermatozoa maintained their motility for extended periods of time and had to be decelerated. They were aspirated by the tail or head and then positioned transversally to the tip of the pipette after re-expulsion. The tip of the pipette was lowered gently, compressing or rubbing the sperm flagellum between the midpiece and tail, which resulted in this part of the sperm sticking to the bottom of the dish. This action destabilized the sperm membrane, impairing motility in a manner similar to prolonged incubation in culture medium or exposure to an acrosome reaction enhancer. It is important to note that motile spermatozoa do not fertilize as well for intracytoplasmic sperm injection as do immotile spermatozoa.

If during the immobilization process the sperm tail was dissected inadvertently or became kinked, the procedure was repeated with another spermatozoon. It is important to emphasize that the presence of too many sperm cells in the PVP-containing droplet may pose a threat to the procedure, as debris carried by the sperm suspension may adhere to the injection pipette and contaminate the injection medium.

At a magnification of 400× it is difficult to select spermatozoa in terms of morphology while they are in motion and without the use of stains. However,

selection of normally shaped spermatozoa can be accomplished to a certain extent by observing their shape, light refraction, and motion patterns.

Once the sperm is immobilized it is crucial to separate it from the bottom of the dish by gently aspirating it by either its head or tail, being careful to avoid kinking the free extremity on the sticky segment. Once loose, the sperm cell should be aspirated tail first. If the spermatozoon moves too rapidly, the pipette is filled with more PVP-containing medium followed by the sperm, once again maintaining a slight proximal motion. The injection needle then should be lifted slightly by turning the knob of the joystick clockwise to avoid damaging the needle spike in case there are any scratches in the dish. The microscope stage is then moved, locating the needle into the oocyte drop. It is important to note, the difference in medium consistency (PVP versus culture medium) may allow the sperm to move distally into the pipette and become loose.

Penetration Into the Ooplasm

Upon focusing on the oocyte, the holding pipette is lowered. Using both tools the oocyte is rotated slowly to locate the polar body and the area of cortical rarefaction (or polar granularity) that occupies a polar segment of the oocyte. This area is presumed to be the site of the germinal vesicle breakdown and consequently the area from which the first polar body is extruded. This area should be avoided during injection because the polar body is not a reliable reference point, as during corona-cumulus cell removal the polar body may roll around within the perivitelline space.

The oocyte is held in place by suction through the holding pipette. By focusing, the equatorial plane of the oocyte is located, subsequently, the depth of the holding pipette is adjusted to have its internal opening also in focus. This allows for greater support of the holding pipette in a position contralateral to the injection point. It is ideal to have the inferior pole of the oocyte touching the bottom of the dish, as it affords a better grip of the egg during the injection procedure. The injection pipette is lowered and focused with the outer right border of the oolemma on the equatorial plane at 3 o'clock. The spermatozoon is then ejected slowly, close to the beveled opening of the injection pipette, and is pushed onto the zona, permitting penetration, and thrust forward to the inner surface of the oolemma at 9 o'clock. At this point, a break in the membrane should occur at the approximate center of the egg. This phenomenon is demonstrated by a sudden quivering of the convexities of the oolemma (at the site of invagination) above and below the penetration point, as well as the proximal flow of the cytoplasmic organelles and the spermatozoon into the pipette. Additional aspiration of cytoplasm is necessary to allow the sperm to move proximally another 60 μm. The cytoplasmic organelles and the spermatozoon then are ejected back into the cytoplasm slowly, to avoid injecting a massive amount of medium; to allow replacement of the spermatozoon, the amount of medium should be kept to an absolute minimum. The aspiration of cytoplasm is necessary to activate the egg and because oocytes have very distinct stages of maturity depending on the time spent in vivo or in vitro, the oolemma may be particularly soft or elastic. In this case it is necessary to withdraw the pipette from the first membrane invagination and slowly repeat the procedure, hooking the upper or lower border of the invagination with the spike to penetrate the oocyte in a line parallel to its equatorial plane. To optimize the interaction between sperm and cytoplasm the sperm should be ejected past the tip of the pipette to ensure a tight insertion among the organelles, which will help maintain the sperm in place while withdrawing the pipette. When the pipette is at the approximate center of the egg some surplus medium is reaspirated so that the cytoplasmic organelles can tighten around the sperm, thereby reducing the size of the breach created during perforation. This also facilitates the closure of the terminal part of the funnel-shaped opening at 3 o'clock. Once the pipette is removed the breach area is observed; the border of the opening should maintain a funnel shape with a vertex into the egg. If the border of the oolemma becomes everted the cytoplasmic organelles can leak out and the oocyte consequently may lyse. The average time required to inject an oocyte was approximately 1 minute.

Evaluation of Fertilization, Assisted Hatching, and Rescue of Poor Quality Embryos

Oocytes were observed 12 to 17 hours after the injection procedure. Integrity of the oocyte cytoplasm and the number and size of pronuclei were noted. Twenty-four hours after fertilization, cleavage of the fertilized oocytes was assessed; for each embryo the number and size of the blastomeres were recorded as well as the percentage of anucleate fragments. Another evaluation of the cleavage was performed after an additional 24 hours and the embryos were screened for the need of assisted hatching. After zona drilling using acidified Tyrode's solution (pH 2.35), an opening in the ZP was made and the anucleated fragments were removed. The procedure was carried out in 10 μL droplets of HTF-HEPES medium supplemented with 15% maternal serum (21). Approximately 72 hours after the microinjec-

tion procedure, good quality embryos were transferred into the uterine cavity carried in HTF medium supplemented with 75% patient serum. The number of embryos transferred was according to the patient's age. When the patient was ≤30 years, two or three embryos were transferred; 31 to 34 years, three embryos were transferred; 35 to 41 years, four embryos were transferred; and ≥42 years, five or six embryos were transferred. These criteria were indicative as they were influenced by availability and quality of the embryos.

Therapeutic Implantation Support

Starting on the day of oocyte retrieval, 16 mg/d methylprednisolone and 250 mg tetracycline every 6 hours were administered for 4 days to all patients. Progesterone administration started on the 3rd day after hCG (25 to 50 mg IM daily) and was continued daily until the assessment of pregnancy (21).

Statistical Analysis

All statistical tests were performed using Statview 512+ (BrainPower Inc., Calabasas, CA) and Microsoft Excel 4.0 (Microsoft Corporation, Redmond, WA) software programs. Analysis of variance procedures and posthoc multiple comparison tests (Bonferroni and Duncan [22]) were conducted using the Statistical Analysis System (SAS Institute, Cary, NC). All statistical tests were carried out two-tailed at the 5% level of significance.

The χ^2 test was used to analyze the fertilization and ongoing pregnancy rates (PRs) for each category of motile spermatozoa found in the ejaculate. The percentage of positive fetal heart beats related to the number of embryos replaced and the number of embryos hatched among the different categories of embryo replacement were analyzed also. Single-factor analysis of variance was used to compare the sperm concentration, motility, and morphology among the different semen samples. Where significant differences were found, multiple comparison tests (Bonferroni and Duncan) were applied to establish which mean differences were significant (22).

RESULTS

The primary clinical indications for infertility involved the male partners' subnormal semen parameters (n = 150) or antisperm antibodies bound to the spermatozoa (n = 4). The indications involving female partners were nonpatent tubes (n = 1), the presence of antisperm antibodies in the serum (n = 5), and mild endometriosis (n = 3). In seven couples a clear reason for the infertile status was not identified. In the remaining 57 couples the presence of male factor infertility was associated with other indications.

Table 1 Survival and Fertilization Characteristics of the Injected Oocytes*

Group	
1. Metaphase II injected	1,923
Survived	1,787 (92.9)
2. Two pronuclei	1,142 (59.4)†
3. One pronucleus	161 (11.7)‡
4. Three pronuclei	78 (5.6)‡

* Values are no. of oocytes with percentage in parentheses.
† Percentages on 1.
‡ Percentages on 2 + 3 + 4.

One hundred seventeen couples were treated unsuccessfully in 322 IVF cycles (the average number of attempts was 2.8). In these previous IVF cycles the failure of fertilization was complete. The mean values of the sperm parameters of these couples were 52.3 × 10^6/mL concentration, 45.6% motility, and 4.8% normal forms. Only 16 of the male partners presented with normal semen characteristics at the time of the attempt and one or more sperm parameters were impaired. The fertilization failure was assumed to be due to the impaired semen functionality not assessable by the routine semen analysis. In fact, 110 couples could not be treated by IVF because of extremely poor semen parameters (mean values of 13.3 × 10^6/mL sperm density, 27.0% progressive motility, and 1.7% normal forms).

All but 142 of 2,473 oocyte-cumulus cell complexes were recorded as mature at the time of oocyte retrieval. After removing the surrounding cumulus and corona cells, 2,417 oocytes (97.7%) revealed an intact ZP and a clear cytoplasm. A total of 1,962 of 2,417 oocytes had extruded the first polar body (81.2%), 10.8% (260/2,417) presented a germinal vesicle, and 8.1% (195/2,417) had undergone germinal vesicle breakdown (32 oocytes were degenerated and 24 were without zonae).

Table 1 describes the number of metaphase II oocytes treated as well as the number of oocytes that survived and fertilized after intracytoplasmic sperm injection. Eight additional oocytes were injected by subzonal insemination in one patient and 24 oocytes in five patients were treated by conventional IVF. Of the intracytoplasmic sperm injection-injected oocytes, three were metaphase I and were matured in vitro overnight and injected the next day after extruding the first polar body. The total number of degenerated oocytes was relatively low (136/1,923; 7.1%).

Of the 1,142 bipronucleated oocytes, 878 embryos were obtained (76.9 %). Of these embryos, 222 had no anucleated fragments; 292 embryos presented be-

Table 2 Fertilization and Pregnancies Obtained After Intracytroplasmic Sperm Injection in Relation to Total Number of Normal Motile Spermatozoa Present in the Ejaculate

Group	No. of motile spermatozoa	No. of cycles	Fertilization rate*	No. of positive hCG	No. of ongoing pregnancies*
1	0	76	354/608 (58.2)*†	38	23 (30.3)
2	39 to 500,000	100	531/810 (65.5)†	61	44 (44.0)
3	>500,000	51	257/369 (69.6)†	25	17 (33.3)
Total		227	1,142/1,787 (63.9)	124	84 (37.0)

* Values in parentheses are percentages.

† 1 to 3: χ^2, 2 × 3, 2 *df*; differences within normal motile sperm concentration on fertilization, $P < 0.001$. Posthoc comparison using Bonferroni adjustment shows no significant difference between the fertilization rates for groups 2 and 3, whereas the fertilization rate for group 1 is significantly different from groups 2 and 3.

tween 5% and 10% anucleated fragments; 183 embryos had between 11% and 20% fragments; and 181 embryos had >20% fragments.

Approximately 72 hours after the intracytoplasmic sperm injection procedure, 653 embryos were transferred in 217 cycles. A total of 516 (79.0%) embryos were micromanipulated for assisted hatching before replacement. Remaining good quality embryos (n = 149) were cryopreserved in 38 patients.

One hundred twenty-four patients presented a positive hCG: 19 pregnancies were biochemical, 10 patients had a blighted ovum, and 1 patient had an ectopic pregnancy requiring a salpingectomy at 7 weeks of gestation. In 94 patients a fetal heart beat was observed on ultrasonographic evaluation; of these couples, 10 miscarried between 7 and 13 weeks of gestation. The ongoing PR was 37.0% per oocyte retrieval (84/227) and 38.7% per replacement (84/217). There were 47 singleton pregnancies (5 of which were vanishing twin pregnancies), 30 twin gestations, 6 triplet pregnancies, and 1 quadruplet pregnancy. The frequency of sac formation per embryo was 24.5% (160/653) and 22.4% (146/653) had a fetal heart beat.

The relation between sperm parameters and the outcome of assisted fertilization was investigated. None of the single sperm parameters such as concentration, progressive motility, or morphology correlated with the outcome of intracytoplasmic sperm injection. A semen score was calculated on the basis of the number of normal motile spermatozoa present in the fresh ejaculate: volume (mL) × concentration (× 10^6/mL) × total motility (%) × normal forms × 10^6. Based on the score, the cycles were divided into three groups: one group with nonmotile spermatozoa in the ejaculate, one with motile spermatozoa ranging from 39 to 500,000, and the last group with >500,000 motile spermatozoa. The first two groups of patients generally are considered not suitable for treatment with standard IVF. Table 2 describes the number of cycles, fertilization rates, and pregnancy results in terms of positive hCG as well as the presence of fetal heart beats at US, according to the concentration of motile sperm cells.

Intracytoplasmic sperm injection can achieve fertilization regardless of motility and sperm concentration of normal motile spermatozoa. Thus, spermatozoa to be used for intracytoplasmic sperm injection can be collected by masturbation, electroejaculation, or microsurgical epididymal aspiration and be processed or cryopreserved for future attempts (Table 3).

To demonstrate the effect of the number of embryos transferred related to embryo implantation, we have analyzed each couple by the number of embryos replaced into the uterine cavity. The number of couples and the total number of embryos transferred, micromanipulated for assisted hatching, and implanted are reported in Table 4.

The high fertilization rates yielded from intracytoplasmic sperm injection appear to be independent of semen characteristics and/or origin. It is interesting to observe the improved therapeutic powers of this technique as compared with conventional IVF performed in the same period of time, in couples with tubal infertility, using the same batches of culture media and identical criteria and laboratory methods (Table 5).

DISCUSSION

In this study 227 couples were treated in 227 consecutive cycles of assisted fertilization by intracytoplasmic sperm injection. Injection of a single spermatozoon into the cytoplasm was performed in 1,923 oocytes, 1,142 of which fertilized normally. The frequency of fetal heart beats per embryo was in excess of 22%. It has been reported in previous papers that patients treated by this technique could indeed achieve pregnancies and give birth to normal babies (10). It also has been shown that intracytoplasmic sperm injection performs consistently better, in terms of fertilization, than subzonal insemination (11, 12). Subzonal insemination requires only a few

Table 3 Semen Parameters, Fertilization, and Pregnancies Obtained With Intracytoplasmic Sperm Injection According to the Origin of the Semen Sample

Semen origin	Density	Motility	Morphology	No. of cycles	Fertilization*	No. of positive hCG	No. of ongoing pregnancies*
	$\times 10^6/mL$		%				
1. Fresh	21.6	34.4†	2.2‡	193	972/1,484 (65.5)§	105	73 (37.8)
2. Frozen	23.9	9.4†	1.6‡	13	75/113 (66.4)§	6	2 (15.4)
3. Electroejaculation	27.0	10.3†	3.8‡	4	23/34 (67.6)§	1	0
4. Epididymal	13.4	16.2†	4.2‡	17	72/156 (46.2)§	12	9 (52.9)

* Values in parentheses are percentages.
† 1 to 4: Single factor analysis of variance, 3 *df*; differences between sperm motility among different semen origin, $P < 0.001$.
‡ 1 to 4: Single factor analysis of variance, 3 *df*; differences between sperm morphology among different semen origin, $P = 0.009$.
§ 1 to 4: χ^2, 2 × 4, 3 *df*; differences within semen origin on fertilization, $P < 0.001$. Posthoc comparison using the Bonferroni adjustment shows no significant difference in motility between groups 2 and 3, and no significant difference between groups 1 and 4. For morphology groups 1, 2 and 3 show no significant difference, whereas group 4 is significantly different from groups 1, 2, and 3. For fertilization percentage, groups 1, 2, and 3 are not significantly different from each other whereas the group 4 percentage is significantly different from the other three rates.

spermatozoa but its success is highly dependent upon the ability of those spermatozoa to fuse with the oolemma and when this happens penetration of more than one sperm cell often occurs, rendering the embryo genetically abnormal (5).

Mechanical damage resulting from the injection procedure was relatively low in this study (136/1,923; 7.1%) as compared with previous reports (11, 12). This is most likely a result of the positioning of the oocyte in relation to the polar body, thereby avoiding the area of polar granularity during the insertion of the injection pipette. This area is considered the place where the germinal vesicle breakdown takes place and where the chromosomes and mitotic spindle are located.

This technique achieves a consistent fertilization rate of 59.4% (63.9% of intact oocytes); in fact, only two couples had a complete failure of fertilization and in one the semen was obtained by microsurgical epididymal aspiration.

The number of motile spermatozoa generally needed to perform standard in vitro insemination is approximately 500,000. Patients with ≥500,000 spermatozoa had a fertilization rate of 69.6% (257/369) with intracytoplasmic sperm injection. When the procedure was performed with samples containing <500,000, the fertilization rate was 62.4% (885/1,418); although significantly lower ($P = 0.01$), the rate was very similar to that obtained using standard IVF in selected patients. When the semen analysis showed no normal motile spermatozoa in the ejaculate, the consequent fertilization rate was 58.2% (354/608). The exclusion of the morphology parameters from the calculation did not make any substantial difference in terms of fertilization and pregnancies. Motility characteristics should be considered those values that were assessed from the initial semen sample and that the processing of the semen by spinning almost always allowed the retrieval of some motile or twitching spermatozoa that ultimately could be used for the injection. These findings demonstrate the consistency of the intracytoplasmic sperm injection technique in achieving fertilization independent of the semen parameters and

Table 4 Number of Embryos Hatched, Replaced, and Implanted in Couples Grouped According to the Number of Embryos Transferred

	Category				
	1	2	3	4	5
Cycles	22	39	79	68	9
Average patients age (y)	35.8	34.9	33.8	36.0	40.6
Embryos replaced	22	80	234	272	45
Embryos hatched	20	61	165	227	43
Sacs	5	12	80	56	7
Fetal heart beats*	2 (9.1)†‡	11 (14.1)†‡	77 (32.5)†‡	52 (19.1)†‡	4 (8.9)†‡

* Values in parentheses are percentages.
† 1 to 5: χ^2, 2 × 5, 4 *df*; differences within embryos groups on implantation in relation to the embryos transferred, $P < 0.001$.
‡ 1 to 5: χ^2, 2 × 5, 4 *df*; differences within embryo groups on implantation in relation to the embryos hatched, $P < 0.001$. Posthoc comparison using the Bonferroni adjustment shows no significant difference among rates for embryos hatched or transferred except for the group representing three embryos transferred, which was significantly different from the other groups for both parameters.

Table 5 Outcome of Couples Treated By Intracytoplasmic Sperm Injection and IVF (Tubal Indication) in the Same Time Period

	Intracytoplasmic sperm injection	IVF
No. of cycles	227	179
No. of inseminated oocytes	1,923	1,719
No. of fertilized oocytes*	1,142 (59.4)	1,086 (63.2)
No. of embryos transferred	653	530
No. of replacements	217	173
No. of ongoing pregnancies*	84 (37.0)	59 (32.9)

* Values in parentheses are percentages.

consonant with a previous study of intracytoplasmic sperm injection (11). The relative unimportance of semen parameters has been presented already by this program (17), however, the fertilization rates obtained by intracytoplasmic sperm injection are three times higher for all subgroups in this current study. The absence of a correlation between sperm characteristics (density, motility, and morphology) and eventual fertilization after intracytoplasmic sperm injection raises the question of whether spermatozoon integrity is indeed necessary to achieve fertilization in the human. A preliminary study has been performed in our laboratory involving intracytoplasmic injection of only the sperm head after mechanical separation of the midpiece and tail. The fertilization rate was similar to the results obtained when the complete spermatozoon was injected; of 13 oocytes injected with sperm heads, 10 showed two pronuclei. This demonstrates that the midpiece is unnecessary for sperm nucleus decondensation, but further experiments are needed to investigate whether embryos will, in fact, develop normally. This is especially important because we recently demonstrated that the first embryonic spindle is organized by the male sperm centrosome, which is located distally in the midpiece section (23).

The absence of a clear difference between the outcome of intracytoplasmic sperm injection in relation to the treatment and collection method of semen has been demonstrated. Spermatozoa collected by epididymal aspiration, which supposedly are less mature, and those exposed to unfavorable conditions during collection by electroejaculation or after cryopreservation performed equally well with intracytoplasmic sperm injection when compared with spermatozoa collected by masturbation.

The incidence of embryo implantation (Table 4) increased progressively when up to three embryos were replaced. Embryo implantation decreased when four and five embryos were transferred and may be explained by the more advanced age of the patients.

The main purpose of this investigation was to demonstrate that the intracytoplasmic sperm injection technique affords consistent and reliable results in terms of fertilization and pregnancies, independent of sperm parameters and/or sperm origin. These results are similar (regarding fertilization and pregnancies) to those obtained after IVF in patients with tubal indication of infertility other than male factor. However, it should be considered that the fertilization rate achieved with intracytoplasmic sperm injection was calculated on the number of metaphase II oocytes injected, whereas in IVF the rates were calculated on the total inseminated oocytes. In this experience, after cumulus removal, 18.8% of the oocytes retrieved were immature.

Of the total number of oocytes displaying pronuclei there was an 11.7% (161/1,381) incidence of oocytes with only one pronucleus versus the 4.7% (78/1,667) obtained after IVF. The embryos obtained from those monopronucleated oocytes are a result of parthenogenetic activation as has been shown by the fluorescence in situ hybridization analysis of the blastomeres of embryos derived from these oocytes (Sultan KM, Munné S, unpublished results). It has been suggested that single pronucleated oocytes can be used in nuclear transplantation (23). Furthermore, the intracytoplasmic sperm injection procedure can be used to inject oocytes that have failed IVF, thus permitting investigation of mechanisms of egg activation and fertilization (24).

Although normal fertilization occurs in approximately 60% of surviving oocytes after intracytoplasmic sperm injection, rare instances of abnormal fertilization may occur. In 5.6% (78/1,381) of activated oocytes, three pronuclei were present. This abnormality, due to failure of second polar body extrusion, can be demonstrated to be digynic by cytogenetic evaluation. Unlike dispermic embryos, digynic zygotes can be rescued by enucleation of the extra digynic pronucleus (25). Correction is ineffective when applied to zygotes displaying three pronuclei (dispermic) because the presence of an extra paternal centrosome induces the formation of abnormal mitotic spindles, resulting in mosaic embryos. These findings further support the new hypothesis on the paternal inheritance of the human centrosome (25).

For the first time we clearly demonstrate that intracytoplasmic sperm injection results in higher fertilization and pregnancy in couples where sperm characteristics are impaired severely and compares favorably to the fertilization and PRs achieved with standard IVF in couples in whom no sperm abnormalities exist. The results suggest that intracytoplasmic sperm injection should be studied in patients with other infertility causes as well.

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