The effectiveness of intracytoplasmic sperm injection combined with piezoelectric stimulation in infertile couples with total fertilization failure

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Objective: To assess the effectiveness of intracytoplasmic sperm injection (ICSI) combined with piezoelectric stimulation in infertile couples with a history of total fertilization failure (TFF).
Design: Prospective controlled trial.
Setting: Clinical IVF laboratory.
Patient(s): Seventy-one couples undergoing ICSI on sibling oocytes having at least one previous ICSI attempt with TFF.
Intervention(s): ICSI or ICSI with piezoelectric activation.
Main Outcome Measure(s): Fertilization rate.
Result(s): The patients were allocated to two groups: group I included 21 patients with only one previous TFF and group II included 50 patients with more than one previous TFF. Collectively, a total of 823 metaphase II (MII) oocytes were retrieved in 78 oocyte retrievals. In Group I, combined ICSI with piezoelectric stimulation was applied to 123/211 (58.2%) of MII oocytes (group IA), whereas standard ICSI procedure was applied to 88/211 (41.8%) of MII oocytes (group IB). The fertilization rate was 62% and 12% in group IA and group IB respectively. In group II, piezoelectric activation was applied in all 612 MII oocytes, of which 296 (48.3%) were fertilized. The rates for implantation and pregnancy/embryo transfer were obtained as 30.6% and 44.1%, respectively.
Conclusion(s): Piezoelectric activation seems to improve IVF outcome in patients with previous TFF history. (Fertil Steril® 2010;94:900–4. ©2010 by American Society for Reproductive Medicine.)

Key Words: ICSI, electrical activation, fertilization rate, embryo grade, pregnancy

Micromanipulation techniques have been used in patients with a history of low fertilization rates or fertilization failure after classical IVF. Intracytoplasmic sperm injection (ICSI) has recently become the standard treatment of infertility resulting from either severe sperm defects or fertilization failure in previous IVF cycles (1). Fertilization rates using ICSI have been reported to be in the range of 50–70% (2, 3). The cause of fertilization failure after ICSI is different from that of conventional IVF. In conventional IVF, 60–90% of the oocytes that are failed to fertilize do not contain sperm nuclei; that suggests penetration failure or sperm ejection as the main cause of fertilization failure (3). In contrast, the main cause of fertilization failure after ICSI appears to be the failure of oocyte activation. Sixty to 70% of unfertilized, metaphase II (MII) oocytes after ICSI contain a swollen sperm head, indicating that the oocyte was correctly injected but failed to become activated to complete the second meiotic division (2–5).

Intracytoplasmic sperm injection has been used widely in the treatment of severe male factor infertility. Many centers have reported fertilization rates of approximately 70%, although most of the sperm injected are deposited into the cytoplasm. Furthermore, when spermatids are used fertilization rates are even lower (6). Many investigators have postulated that the major reason for failed fertilization after ICSI might be suboptimal stimulation of inositol 1,4,5-triphosphate–induced calcium release and/or calcium-induced calcium release as a result of insufficient release of the soluble proteins from the injected sperm heads (7).

Yanagida et al. (8) was the first to use piezo electric stimulation for human oocyte activation that resulted in a live twin birth. Animal studies have shown that an electrical field can generate micropores in the cell membrane of gametes and

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somatic cells to induce sufficient Ca^{2+} influx through the pores to activate cytoplasm by means of Ca^{2+}-dependent mechanism (9). Previous studies reported that mouse oocytes injected with secondary spermatocytes or spermatids were fertilized when stimulated by electroporation and developed into normal offspring when the resultant embryos were transferred to a recipient uterus (10–12). The aim of this study was to assess the use of ICSI combined with piezoelectric stimulation in infertile couples having a history of total fertilization failure (TFF) in previous ICSI cycles.

MATERIALS AND METHODS

The current study included 71 patients with unexplained infertility undergoing IVF using ICSI at Gen-Art Woman Health and Reproductive Biotechnology Center between January 2005 and September 2007. Collectively, 823 MII oocytes were analyzed in 78 IVF cycles. All of the patients had at least one fertilization failure in previous IVF attempts, and all patients were comprehensively informed about the novelty of the procedure. They were fully aware of the limited data available regarding the effectiveness and safety of the procedure and signed an appropriate written consent for the use of piezoelectric with ICSI.

Of the 71 patients, 21 of them had only one previous TFF experience (group I). In order to evaluate the recurrence risk of their fertilization failure, the oocytes of each patient were randomly allocated after ICSI into two pilot groups: study group (group IA [piezoelectric activation]) and the control group (Group IB [without piezoelectric activation]). Oocytes from patients with >1 previous TFF all underwent ICSI with piezoelectric activation (group II). In group I we evaluated only embryology data, whereas in Group II both embryology data and clinical outcomes were assessed.

Ovarian Stimulation

Ovarian suppression was achieved either by GnRH analogs (Decapeptyl; Ferring, Hoofddorp, The Netherlands) started at the midluteal phase of the menstrual cycle, or GnRH antagonists (Cetrotide; Serono, Benelux, The Netherlands) started when the leading follicle was >13 mm in diameter or when the serum estradiol level was >350 pg/mL. For ovarian stimulation recombinant FSH (follitropin alpha [Gonal F, Rome, Italy] or follitropin beta [Puregon; Schering Plough, Berlin, Germany]) or purified urinary menotropins (Menogon; Ferring, Kiel, Germany) were used. Starting dose was decided by age, body mass index, day 3 hormonal values and antral follicle count, and previous response to ovarian stimulation. For luteal phase supplementation, micronized progesterone was given intravaginally (200 mg three times per day).

Semen Preparation

Semen samples were prepared with a discontinuous gradient method and a swim-up method according to sperm concentration (13, 14). Semen parameters of these patients were $>20 \times 10^6$ sperm/mL (normospermia).

Oocyte Retrieval and Preparation

The culture medium used for oocytes and embryos was Vitrolife (IVF Science, Göteborg, Sweden). Microinjection was performed 2–4 hours after oocyte pick-up as described previously (1, 2). A high number of oocytes were assigned to ICSI with piezoelectric activation to secure fertilization, because not every oocyte can be injected (approximately 5–10% because of their maturational stage) or will survive after injection (approximately 3%). Oocytes were checked for the presence of pronuclei and polar bodies 16–18 hours after ICSI. Routine examination of embryo quality included the number of blastomeres, the degree of fragmentation, and the uniformity of the blastomeres. Collected oocytes were placed in G-Fert (Vitrolife; IVF Science) droplets, and incubated in a humidified 37°C incubator in 6% CO_2 in air for 2–4 hours. Cumulus and corona cells were removed enzymatically in Hyase10X (Vitrolife; IVF Science, Sweden) for a maximum of 30 sececonds. The denuded oocytes were examined to assess integrity and maturity. Only those oocytes that extruded the first polar body (MII oocytes) were microinjected. Oocytes were microinjected in microdroplets of G-MOPS (Vitrolife; IVF Science) medium.

Piezoelectric Stimulation of Injected Oocytes

Piezoelectric stimulation was applied 20 minutes after sperm injection. Electroactivation was performed using a BTX Electro-cell manipulator (BTX, San Diego, CA) at room temperature with a chamber with two stainless steel electrodes 0.5 mm apart, filled with activation buffer (pH = 7.0) including Mannitol (0.3 M), $MgSO_4$ (0.1mM), $CaCl_2$ (0.1 mM), HSA (0.05 mg/mL), and HEPES (0.5 mM). Injected oocytes were activated with a single pulse of 1.5 kV/cm DC for 100 μsec. Stimulated oocytes were immediately transferred back to culture medium—G1 (Vitrolife; IVF Science) droplets in a humidified atmosphere of 6% CO_2 at 37°C.

Assessment of Fertilization

Fertilization was assessed 12–16 hours after microinjection. The injected oocytes were observed for any sign of damage and for the presence of pronuclei. Oocytes were classified as fertilized if two pronuclei (PN) were present. Abnormally fertilized oocytes (1PN or 3PN) were excluded. Normally fertilized oocytes were left in culture for another 24 hours. Embryos were classified according to a simplified system based on Veeck's morphological criteria: Grade I embryos have equal-sized blastomeres and no cytoplasmic fragmentation, grade II embryos have blastomeres of equal size and minor cytoplasmic fragmentation covering $\leq$10% of the preembryo surface, grade III embryos have blastomeres of distinctly unequal size and variable fragmentation, grade IV embryos have blastomeres of equal or unequal size and moderate-to-significant cytoplasmic fragmentation covering >10% of the preembryo surface, and grade V embryos have few blastomeres of any size and severe fragmentation covering $\geq$50% of the preembryo surface. None of the embryos were classified as grade V in this study.

Embryo Transfer

Embryos were transferred on the third day after oocyte retrieval. The best-quality embryos were transferred, regardless of whether they were derived from ICSI with piezoelectric activation or from standard ICSI. Depending on the woman's age and the embryo quality, one to three embryos were transferred. All embryos were transferred using an Edwards Wallace catheter (Simcare Ltd., West Sussex, United Kingdom). Embryo transfers were performed using transabdominal sonographic guidance. Progesterone capsules (600 mg per day) were administered intravaginally to all patients beginning 2 days after oocyte retrieval. Biochemical pregnancy was established when serum β-HCG was found >20 IU/L on the 12th day after embryo transfer, and clinical pregnancy was defined as the presence of a gestational sac on ultrasound examination at 6 week's gestation. The implantation rate was defined as the ratio of the number of gestational sacs divided by the number of embryos transferred. Ongoing pregnancy was defined by the presence of an embryo with a heart beat beyond 12 week's gestation.

Statistical Analysis

Data analysis was performed by using SPSS for Windows, version 11.5 (SPSS, Inc., Chicago, IL). Whether the distributions of continuous variables were normal or not was determined by using Shapiro-Wilk test. Data were shown as mean $\pm$ SD for continuous variables and percentages for categorical data. Means were compared using analysis of variance when the continuous variables were normally distributed, otherwise groups were compared using the Kruskal-Wallis test. When the P value from the Kruskal-Wallis test statistics is statistically significant, Kruskal-Wallis multiple comparison test was used to know which groups differ from which others. Categorical variables were evaluated by χ^2 or Fisher's exact test, where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 71 infertile couples with TFF were enrolled. Collectively, 823 MII oocytes were retrieved in 78 oocyte retrievals. Table 1 shows demographic and clinical characteristics of the patients in both groups. Group I and group II were comparable in regard to mean age, body mass index, and day 3 serum FSH levels. Day 3 serum estradiol was lower in Group I compared with group II (44.7 $\pm$ 18.03 vs. 66.7 $\pm$ 9.79; $P > 0.001$), and the duration of infertility was longer in group I compared with group II (6.9 $\pm$ 2.1 vs. 5.5 $\pm$ 1.6, respectively; P=0.003).

In group I, combined ICSI with piezoelectric stimulation was applied to 123 MII oocytes (group IA), whereas standard ICSI was applied for the remaining 88 (group IB). In group II, all of the oocytes were subjected to ICSI with piezoelectric activation. In group IA 76 of 123 oocytes (62%) were fertilized, whereas in group IB 10 of 88 oocytes (12%) were fertilized (P=0.001). When embryos were graded, 28/76 (37%) embryos in the piezo group (group IA) and 2/10 (20%) embryos in the control group (group IB) were assigned as grade 1–2 (P=0.01), whereas 48/76 (63%) embryos in group IA and 8/10 (80%) in group IB were classified as grade 3–4 (P=0.01; Table 2).

Piezoelectric stimulation was performed on all oocytes of the patients having >1 previous TFF experience. Six hundred and twelve MII oocytes from 50 infertile couples were piezo activated and 296 oocytes were fertilized (49%). When grades of the embryos were evaluated, 103 embryos were assigned as grade 1–2 (35%) and 193 embryos were assigned as grade 3–4 (65%). The implantation rate, pregnancy rate/cycle, pregnancy rate/embryo transfer, and ongoing pregnancy rate/embryo transfer were obtained as 30.6%, 41.7%, 44.1%, and 42.8% respectively (Table 3).

DISCUSSION

In order to increase pregnancy rates, various strategies have been developed. Even though limited data are available, initial studies reported that use of ICSI combined with electrical stimulation in patients with failed fertilization resulted in increased fertilization rates (8, 9). Here we present a novel strategy in which oocyte activation was achieved through piezoelectric application after ICSI.

Zhang et al. (10) reported that electrical activation of oocytes that failed to fertilize after ICSI could resume apparently normal fertilization and early embryonic development. However, their stimulation protocol was more complicated than ours, as they initially used an alternate current electrical pulse of 8 V for 6 seconds to stimulate the oocytes in all

TABLE 1

Baseline characteristic of the patients in Groups I and II.

Variable	Group I (n = 21)	Group II (n = 50)	*P* value (Student's *t* test)
Age (y), mean $\pm$ SD	33.2 $\pm$ 3.1	32.8 $\pm$ 4.0	0.683
Body mass index (kg/m^2)	21.9 $\pm$ 1.7	21.8 $\pm$ 1.7	0.822
Duration of infertility (y)	6.9 $\pm$ 2.1	5.5 $\pm$ 1.6	0.003
Day 3 serum FSH (IU/L)	6.8 $\pm$ 2.15	7.3 $\pm$ 2.13	0.371
Day 3 estradiol (pg/mL)	44.7 $\pm$ 18.03	66.7 $\pm$ 9.79	<0.001

Baltaci. ICSI combined with electrical stimulation. Fertil Steril 2010.

TABLE 2

Fertilization and embryo grade results after ICSI with piezoelectric activation or conventional ICSI for patients having one previous TFF experience (group I).

	Group IA Piezo (+) (n = 123)	Group IB Piezo (–) (n = 88)	*P* value
Oocytes fertilized, n	76	10	
Fertilization rate, %	62	12	0.001
Grade 1–2 embryos, n (%)	28 (37)	2 (20)	0.01
Grade 3–4 embryos, n (%)	48 (63)	8 (80)	0.01

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groups, including the control group, before using either one or three direct current pulses to stimulate the oocytes in the study group with a voltage of 1.36–1.50 kV/cm for 40–60 μsec for each pulse. In the group using three pulses, the interval between each electrical pulse was 15–20 minutes. Those researchers reported fertilization rates of 70%, 78%, and 27% for 1 pulse, 3 pulses, and the control group, respectively (10). The rate of degeneration caused by piezoelectric stimulation in the current study seems higher (12%) compared with a previous study reported by Mansour et al. (5.9%) (9). Patient selection criteria and different pulses and frequency of electrical stimulation used in the studies might have caused this discrepancy between the studies.

Yanagida et al. (8) reported the use of combined ICSI with electrical stimulation in three couples with prior total failed fertilization after ICSI. In that study, oocytes were stimulated with square direct current electrical pulse 30 minutes after ICSI. The researches reported the birth of dizygotic twins in one of the three couples. The twin birth was uncomplicated, with normal karyotypes of 46XX and 46XY. Electrical stimulation of oocytes was performed 20 minutes after ICSI in this study. Tanaka et al. (15) also reported 17 healthy infants born after ICSI with electrically stimulated oocytes using both alternate and direct electrical current. According to those studies, electrical current seemed not to have an unfavorable effect on the resulting embryo, though the sample size was small. Further investigations are required on the effect of electrical current on the genetic composition of embryos before using this stimulation protocol for rescuing unfertilized oocytes for clinical use.

TABLE 3

In vitro fertilization characteristics in women with >1 TFF history.

Parameter	Value
MII oocyte, n	612
Fertilization rate, %	48.3 (296/612)
Oocytes degenerated due to piezo, n (%)	73 (12.0)
Oocytes unfertilized, n (%)	243 (39.7)
Grade I-II embryos, n (%)	103 (35)
Grade III-IV embryos, n (%)	193 (65)
Number of transfers	47
Number of embryo transferred	132
Implantation rate, %	30.6
Pregnancy rate/cycle, %	41.7
Pregnancy rate/embryo transfer, %	44.1
Ongoing pregnancy rate/embryo transfer, %	42.8

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Total failure or limited fertilization after microinjection in normospermia cases have been previously reported (13, 14, 16). In this study, favorable fertilization, implantation, and pregnancy rates were obtained in the patients who had previous unsuccessful IVF attempts as a result of fertilization failure. Most of the TFF cases arise because of the failure in oocyte activation (17). Zhang et al. (10) demonstrated that electrical activation can be used to facilitate fertilization and early embryonic development after ICSI. In addition, it was reported that oocyte activation enabled normal fertilization and pregnancy (18). Mansour et al. (9) reported that oocyte electroactivation after ICSI improved the fertilization rate in severe oligoasthenoteratospermia and nonobstructive azoospermia cases. An electroactivated group and a control group were included in that study and fertilization rates were significantly improved in the electroactivated group (from 60–68%).

Our study results are in agreement with the previous findings. On one hand, significant improvement in fertilization rates was achieved by means of piezoelectric activation that was used along with ICSI in both groups. Alternatively, piezoelectric stimulation generated a higher percentage of top quality embryos with favorable pregnancy rates as opposed to non electric stimulated classical ICSI.

In this study, there was a tendency toward an increase in fertilization and embryo grade rates as a result of ICSI with combined electrical activation. Various studies have shown that electrical activation after ICSI increases fertilization rate and formation of embryo (19). Chang et al. (20) demonstrated that pores on the cell membranes of human red blood cells with electron microscopy as early as 3 μsec after an electrical pulse and that resealed several seconds afterward. They called

this process *electrocorporation*, which transiently permeabilizes the cell membrane. Another study on sea urchin eggs during the course of electroporation showed that there was a transient Ca^{2+} influx during the increase in membrane conductance during electrocorporation (21).

In this prospective controlled study that was planned to assess the effectiveness of ICSI combined with piezoelectric stimulation in infertile couples with prior TFF, we observed that electrically activated oocytes were capable of developing into good quality, healthy embryos that are competent to establish ongoing pregnancies. All of the enrolled patients did not have the chance of pregnancy and even the possibility of embryo transfer in the previous cycles. The preliminary findings showed that the adjunctive of piezoelectric stimulation may both increase fertilization rates and improve embryo grades leading to successful pregnancies in ICSI cycles. However the definitive effect of this novel procedure on developing embryos is currently not known, and both the clinical effectiveness and safety of the procedure should be clarified in further studies.

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