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The usefulness of a piezo-micromanipulator in intracytoplasmic sperm injection in humans

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Intracytoplasmic sperm injection (ICSI) has wide clinical application. In order to achieve good results with this method, it is important to restrict the possibility of oocyte injury as much as possible, and securely inject spermatozoa into the ooplasm. For this purpose, we clinically applied piezo-ICSI, which employs a micromanipulator with piezo-electric elements, to humans, and compared the results with those obtained by conventional ICSI. Conventional ICSI and piezo-ICSI were used in 279 cycles and 335 cycles respectively. Piezo-ICSI showed significantly more favourable results, with a survival rate of 88.1% (conventional ICSI: 81.4, $P < 0.001$), a fertilization rate of 79.4% (conventional ICSI: 66.4%, $P < 0.001$), and a pregnancy rate of 23.1% (conventional ICSI: 14.9%, $P < 0.05$). In piezo-ICSI, the needle used is not sharpened and has a flat tip. However, deformation of the oocyte during insertion of the needle is restrained by vibration of the piezo, and the oolemma is punctured readily and securely by the piezo pulse, at the site where the spermatozoon is injected. Piezo-ICSI is a promising new technique for human ICSI that should improve the survival, fertilization and pregnancy rates after ICSI.

Key words: fertilization/intracytoplasmic sperm injection/piezo-micromanipulator/pregnancy

Introduction

Intracytoplasmic sperm injection (ICSI) has been used throughout the world since Palermo *et al.* (1992) introduced its clinical application. However, the survival rates and fertilization rates of oocytes following ICSI are still variable among institutions. Reported survival rates have ranged from 80 to 90%, and fertilization rates of intact oocytes have ranged from 45 to 70% (Payne *et al.*, 1994; Sherins *et al.*, 1995; Tucker *et al.*, 1995; Van Steirteghem *et al.*, 1996). Such dispersion seems to be influenced by the size and sharpness of the needle used for injection (Vanderzwalmen *et al.*, 1996), and by the method for insertion of the needle into ooplasm. Recently, Kimura and Yanagimachi (1995) reported the application of piezo-ICSI to mice in which a micromanipulator applying piezoelectric elements was employed. They achieved high survival and fertilization rates in mice by piezo-ICSI compared with those obtained by conventional ICS, which was characterized by low survival and fertilization rates. In this report, we applied piezo-ICSI to human ICSI to examine whether the results of ICSI would improve.

Materials and methods

Patients

This study includes 614 cycles in patients who had been treated at the Fukushima Medical College Hospital between April 1995 and April 1997. ICSI was conducted on patients with severe male factor and those with fertilization disorders who could not obtain fertilized oocytes more than once by in-vitro fertilization (IVF). Patients with necrozoospermia and azoospermia were excluded. ICSI by the conventional method was used in 279 treatment cycles between April 1995 and January 1996, and piezo-ICSI was used in 335 treatment cycles between January 1996 and April 1997. For each ICSI, the survival rate, fertilization rate, cleavage rate, embryo quality and pregnancy rate after ICSI were compared and examined. ICSI was performed with the approval of the Ethics Board of our institution.

Preparation of oocytes and spermatozoa

Gonadotrophin-releasing hormone agonist (GnRHa) (Suprecur: Hoechst Marion Roussel, Tokyo, Japan) was administered at 900 μg per day from day 21 of the previous cycle (long protocol), and stimulation was provided in treatment menstrual cycles using pure follicle stimulating hormone (FSH) and human menopausal gonadotrophin (HMG). Human chorionic gonadotrophin (HCG) 10 000 IU was administered when the maximum diameter of follicles reached 17 mm, and the oocytes were collected 35 h later. The oocytes were cultured in human tubal fluid (HTF: Irvine Scientific Co., Santa Ana, USA) containing 6% plasmanate cutter (PPF: Bayer Yakukin Ltd, Osaka, Japan) for 3–8 h before ICSI. After that, pipetting was conducted in HEPES-buffered HTF (HEPES-HTF, sperm washing medium, Irvin Scientific Co.) containing 0.025% hyaluronidase (type VIII, Sigma Chemical Co., St Louis, MO, USA) to remove cumulus cells, and spermatozoa were injected into metaphase II oocytes only.

After semen was liquefied for ~30 min, as many motile spermatozoa were recovered as possible by the swim-up method using HTF. When the swim-up method was impossible, the sperm suspensions were prepared by centrifugal washing (250 $g \times 10$ min) using HEPES-HTF.

Instruments

An inverted microscope (IX-70: Olympus, Tokyo, Japan) equipped with Hoffman modulation optics (Hoffman Modulation Contrast, Model EP: Olympus) was used. For the microinjector (IM-4B: Narishige, Tokyo, Japan), a gas-tight syringe (#1750-LT: Hamilton, USA) was used, to which a 21 gauge needle with a rounded tip was attached. A polyethylene tube (external diameter 1 mm: PE-90, Clay Adams Intradermic Inc., Sparks, MD, USA) was connected directly

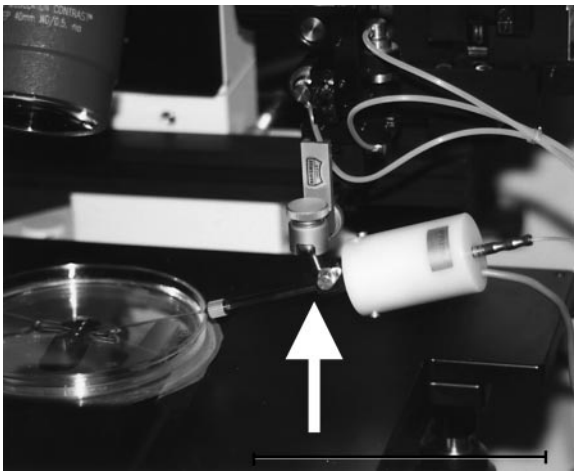

Figure 1. Photograph of the piezo-micromanipulator. The needle holder (manufactured by Leitz co. op.) was fixed to a piezo drive unit (type MB-A). MB-A- and MB-U-type piezo drive units were found to be suitable for piezo ICSI, while the MB-B-type unit was not. The arrow indicates the junction of the needle holder and the micromanipulator. This junction was not fixed, enabling the needle holder to slide using piezo pulses. Scale bar = 10 cm.

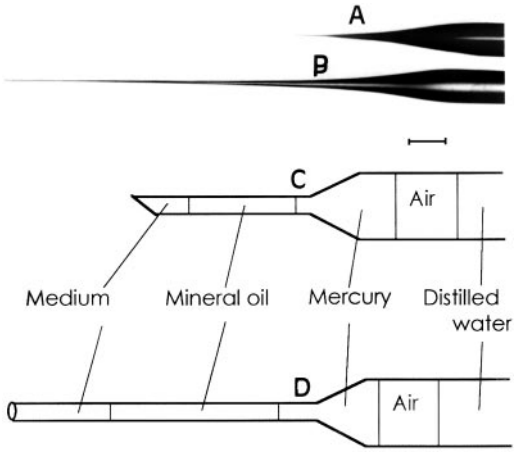

Figure 2. Photographs and illustrations of the injection needles for ICSI. (**A**) and (**C**), conventional ICSI; (**B**) and (**D**), piezo-ICSI. The outer diameter of the glass capillaries was 1 mm. The outer diameter of the tip of both needles was 5–6 μm. The point of the needle for piezo-ICSI was flattened (**D**). (**C**) and (**D**) shows the arrangement at the end of ICSI preparation comes to an end. The medium never comes in contact with mercury (**C**, **D**). Scale bar (**A**, **B**) = 1 mm.

to the needle through a needle holder (Figure 1). The tube was filled with distilled water. The needle holder for injection was attached to the drive unit of a piezo-micromanipulator (PMM-MB-A: Prime Tech Ltd, Tsuchiura, Ibaragi, Japan) was used. The drive unit of the piezo-micromanipulator was driven by a controller (PMAS-CT-140: Prime Tech Ltd).

Preparation of needle

A sterilized glass capillary (borosilicate glass, B100–75–10: Sutter Instrument Co., Novato, CA, USA) was pulled with heating (setting: ramp value; 714, conventional ICSI; Heat = 675, Pull = 93, Vel = 20: piezo-ICSI; Heat = 727, Pull = 50, Vel = 130) using a micropipette puller (Model P-97IVF, Sutter Instrument Co.). The tip of the needle dissolved with 25% hydrofluoric acid (HF; WakoJyunyaku Co., Osaka, Japan). The HF treatment was as follows. A 21 gauge needle was attached to a 10 ml disposable syringe, and to the needle a 3–4 cm polyethylene tube was connected. The needle made by the puller was attached to the tube and the tip of the needle was then immersed in 25% HF while pressurizing the piston of the syringe to extrude 2–3 ml of air, and generation of air bubbles from the tip was confirmed. Once bubbles were produced, pressurizing was suspended, then bubble production was resumed. This operation was repeated rhythmically several times (four to six times) to thin the wall of the needle tip. After that the needle was washed using a similar pumping method in distilled water and 100% ethanol. Then, small amounts (2–4 pl) of mercury (Wako Jyunyaku Co.) were injected into the needle from the opposite side of the tip, and moved toward the tip by pressurization from the syringe. In the same way, the pipette for oocyte holding was made as follows: a glass capillary was pulled with heating (setting; ramp value; 714, Heat = 675, Pull = 95, Vel = 130), the tip of which was shaped to 80–100 μm in external diameter, and 10–15 μm in internal diameter, using a microforge (MF79: Narishige). Figure 2 shows the photographs and illustrations of injection needles for ICSI.

Preparation of chamber

The lid of a plastic Petri dish (90×15 mm: Terumo, Tokyo, Japan) was used for the chamber. Drops of 8% PVP (polyvinyl pyrrolidone, MW 360 000, Sigma) diluted with Dulbecco's phosphate-buffered saline (Gibco, NY, USA), HEPES-HTF and sperm suspension were placed linearly in the chamber, which was covered by mineral oil (M-8410, Sigma). The volume of drops was ~5–10 μl.

Method of conventional ICSI

Just before ICSI, air contained at the tip of the injection needle was extruded as much as possible. After that, small amounts of mineral oil were sucked into the needle, then small amounts of HEPES-HTF were sucked in. Next, a motile spermatozoon was taken into the injection needle from drops of the sperm suspension. The needle was transferred into 8% PVP drops. The spermatozoon was immobilized by pipetting, and injected in drops of the HEPES-HTF. Injection of the spermatozoon was performed after suction of a small amount of ooplasm to ensure penetration of the oolemma (Figure 3).

Method of piezo-ICSI

Just before ICSI, air contained at the tip of the injection needle was extruded as much as possible. After that, small amounts of mineral oil were sucked into the needle, then small amounts of HEPES-HTF were sucked in. Next, the injection needle was inserted into the inner hole of the oocyte-holding pipette under the microscope, which, according to the breaking method, was broken where the outer diameter of the injection needle was 5–6 μm. If the tip was not broken evenly by this operation, it was broken again. A captured motile spermatozoon was immobilized by giving piezo pulses to sperm tails in 8% PVP drops. An immobilized spermatozoon was sucked into the injection needle by its tail and transferred into the HEPES-HTF, after which ICSI was conducted. At first, the needle was allowed to penetrate only the zona pellucida while piezo pulses (five to 10 pulses, about 0.5 Hz) were applied. Then the needle was allowed to penetrate deeply into the ooplasm without applying piezo pulses, and when the oolemma extended sufficiently it was punctured with one piezo pulse. The puncture of the oolemma was confirmed by the ability of the oolemma, which had been brought into the oocyte as the needle was inserted into the oocyte, to return to its original position. No suction of ooplasm was performed during the injection of a spermatozoon (Figure 4).

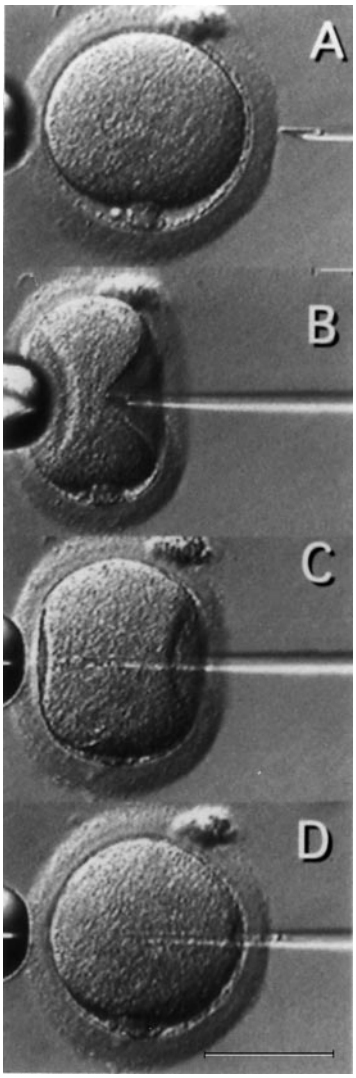

Figure 3. Photographs of conventional ICSI. Photographs were prepared from video tape. Conventional ICSI: an injection needle was inserted into the centre of an oocyte (**A**). The needle was inserted deeply, but the zona pellucida and oolemma were not broken. The oocyte was greatly deformed (**B**). The needle ruptured the oolemma (**C**). A small amount of cytoplasm was sucked into the injection needle to ensure that the membrane was ruptured (**D**). Scale bar = 100 μm.

Culture for oocytes after ICSI

The oocytes treated by ICSI were cultured, and fertilization was assessed 16–18 h later. The quality of the embryos was assessed 48 h after the collection of oocytes in accordance with the classification by Veeck (1991), then up to three of the best embryos (judged by their appearance) were transferred into the uterus.

Luteal support and judgement of pregnancy

Following the embryo transfer, luteal support (progehormone 50 mg/day, Mochida Pharmaceutical Co., Ltd., Japan) was administered for 10 days, and pregnancy was confirmed by detecting an increased urine HCG concentration 14 days after embryo transfer.

Measurement of mercury in the injection drops

We examined whether mercury dissolved in the injection drop. The volume of the injection drop was 5–10 μl, and was too small for measurement of the mercury concentration. Therefore, larger drops of 100 μl were prepared in the chamber. Fifty unfertilized mouse oocytes were injected with human spermatozoa in the same drop by the same manner used for human piezo-ICSI. Ten injection drops in the chambers were collected to the sampling tube (1.5 ml), with precautions taken to prevent contamination. The mercury concentration in the mHTF was measured by atomic absorption spectrophotometry.

Statistical significance was assessed using the χ^2 analysis test. At $P < 0.05$, the difference was considered statistically significant.

Results

Table I lists the results of 279 treatment cycles and 335 treatment cycles treated by conventional ICSI and piezo-ICSI respectively. 1891 oocytes were collected for piezo-ICSI [1629 oocytes (86.1%) of which were in metaphase II] and were treated by piezo-ICSI; 1435 oocytes survived (88.1%, conventional ICSI: 66.4%, $P < 0.001$); 18 h later, 1139 oocytes were fertilized. The fertilization rate for surviving oocytes was 79.4% (conventional ICSI: 66.4%, $P < 0.001$). At 48 h after collection of oocytes, 83.1% (conventional ICSI: 83.0%, NS.) of fertilized oocytes had developed. At that time, an average of 2.2 embryos (conventional ICSI: 2.0 oocytes) was transferred to each patient, and 72 patients became pregnant. The pregnancy rate per embryo transfer was 23.1% (conventional ICSI: 14.9%, $P < 0.05$).

Figure 5 shows the developmental stage of fertilized oocytes at embryo transfer. Most oocytes were at the four-cell stage, which accounted for 59.6% for piezo-ICSI (conventional ICSI: 60.0%, NS). No significant difference was recognized between the two groups at each stage. In accordance with the morphological evaluation of embryos at embryo transfer (Figure 6), oocytes at grade 3 accounted for 27.7% of piezo-ICSI cases, which was significantly higher than that for conventional ICSI ($P < 0.05$). No significant difference was recognized between other grades.

The mercury concentration in the injection drop was 0.6 μg/l in the piezo-ICSI (Table II). When piezo-ICSI was performed without using mineral oil between mercury and mHTF at the tip of the injection pipette, the concentration was 0.4 μg/l.

Discussion

To achieve fertilization by ICSI, it is important to inject immobilized motile spermatozoon securely into the ooplasm (Hoshi *et al.*, 1995; Palermo *et al.*, 1996; Vanderzwalmen *et al.*, 1996). This seems to be related to differences in fertilization results for ICSI among institutes. Experience in the use of ICSI is a very important factor in improving the results. Therefore, we cannot compare the results of ICSI in different periods. In our hospital, conventional ICSI was performed from February 1992; it was thought that the method of conventional ICSI had become well established by 1995 when this research was started. We therefore tried to compare the results of different methods of ICSI, in different periods.

In conventional ICSI, an injection needle penetrates the oolemma through the zona pellucida, which causes considerable deformation of the zona. This deformation may increase

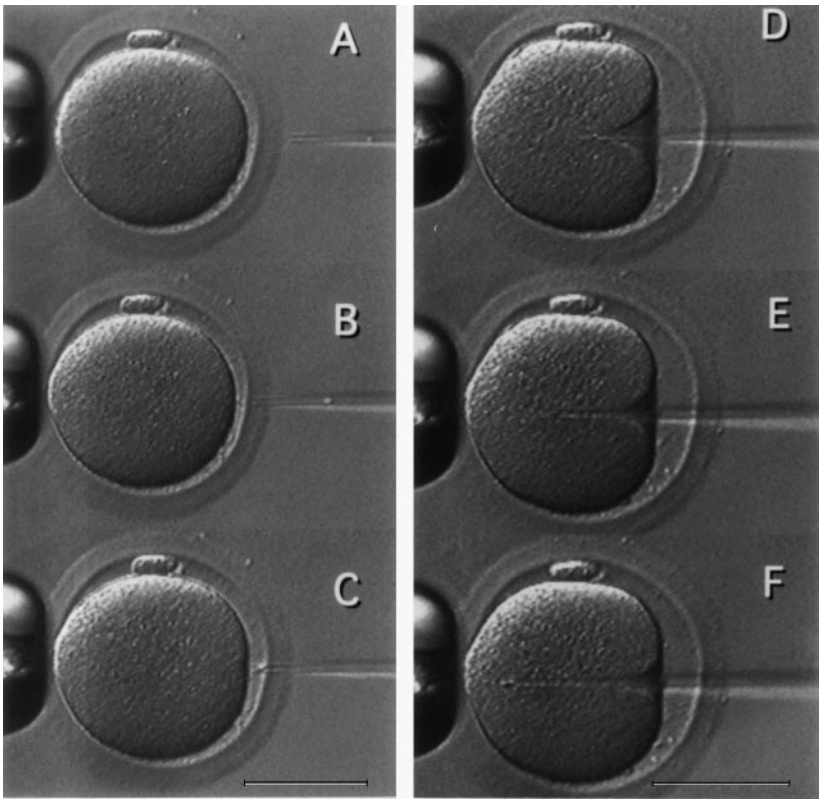

Figure 4. Photographs of piezo-ICSI. Photographs were prepared from video tape. Piezo-ICSI: The injection needle smoothly penetrated the zona using several piezo-pulses (**A**, **B**, **C**). The needle was deeply inserted into the cytoplasm without a piezo-pulse (**D**). Then a single pulse was applied to break the oolemma and rapid relaxation of the oolemma was observed (**E**). A single immobilized spermatozoon was then expelled into the ooplasm (**F**). It was clear that the oocyte was less deformed in the piezo-ICSI than in the conventional ICSI (**D** versus Figure 3B). Scale bars = 100 μm.

Table I. Results of piezo-ICSI using the different methods

	Conventional ICSI	Piezo-ICSI
No. of patients	197	234
No. of treatment cycles	279	335
Age (mean ± SD, years of age)	33.6 ± 4.2	35.0 ± 4.5
No. of oocytes retrieved	1701	1891
Mean no. oocytes retrieved per patient	6.4 ± 3.9	5.9 ± 3.7
Mean no. of injected oocytes[a]	1510	1629
Mean no. oocytes injected per patient	5.4 ± 3.5	4.9 ± 3.2
Surviving oocytes	1229 (81.4)	1435[e] (88.1)
Fertilized oocytes[b]	816 (66.4)	1139[e] (79.4)
Cleaved oocytes[c]	677 (83.0)	947[f] (83.1)
No. of embryos transferred[d]	249 (89.2)	312 (93.1)
Mean no. of embryos per transfer	2.0 ± 1.1	2.2 ± 1.0
% pregnancies per embryo transfer	37 (14.9)	72[g] (23.1)

Values in parentheses are percentages.
[a]All metaphase II oocytes among the oocytes retrieved were treated with ICSI.
[b]Fertilized oocytes as percentage of surviving oocytes.
[c]Cleaved oocytes as percentage of fertilized oocytes.
[d]Embryos transferred as percentage of no. of treatment cycles.
[e]$P < 0.001$, [f]N.S., [g]$P < 0.05$, compared with conventional ICSI, χ^2 test.

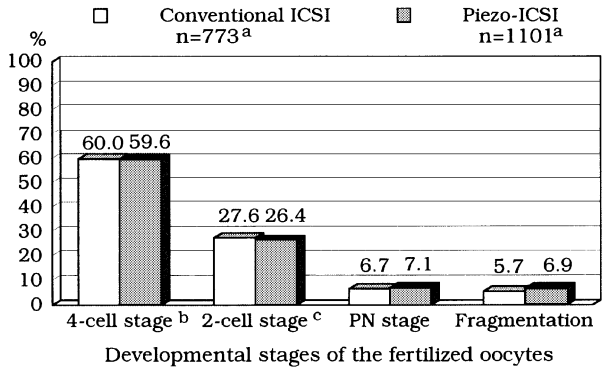

Figure 5. Status of embryos 48 h after oocyte retrieval. No significant differences between any developmental stages. [a]n = no. of fertilized oocytes–no. of cryopreserved oocytes. [b]Embryos with more than four cells were included in the four-cell stage. [c]Embryos with two and three cells were prescribed in the two-cell stage.

the internal pressure of the oocyte, induce the emission of ooplasm from the oocyte after extraction of the needle, and contribute to oocyte death. Since oolemma has high extendibility, the inserted needle may not penetrate the oolemma. Thus, the spermatozoon should be injected after the response of penetration into the oolemma is confirmed. This response has been confirmed by observation of the oolemma, which is pushed into the oocyte as the needle is inserted, then returns to its original position upon penetration. When this response cannot be obtained, the spermatozoon is injected after small amounts of ooplasm are sucked into the needle. On the other hand, the first report on piezo-ICSI was published by Kimura and Yanagimachi (1995), who used mice as subjects. By their method, oolema deformation by change in voltage of piezoelectric elements is achieved by the vibration of piezoelectric elements, and the zona pellucida can be penetrated

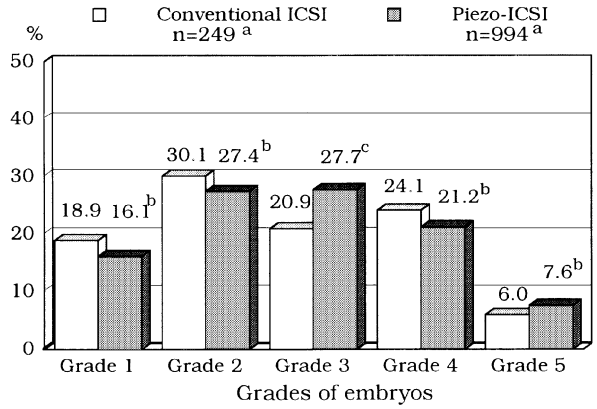

Figure 6. Quality of embryos following ICSI. Quality was evaluated 48 h after oocyte retrieval. Embryo quality was classified from grade 1 to 5 according to Veeck's classification (1991). Grade 1 was the embryo of highest quality, and grade 5 indicated severe fragmentation involving >75% of oocytes. There was no significant difference between the two groups in the number of good quality embryos (grade 1 + grade 2). [a]Numbers of cleaved oocytes checked. [b]Not significantly different compared with conventional ICSI, χ^2 test. [c]$P < 0.05$: compared with conventional ICSI, χ^2 test.

Table II. Mercury concentration in the injection drops

	No. of experiments	No. of drops per experiment	Mercury concentration (μg/l)
Piezo-ICSI[a]	1	10	0.6
Piezo-ICSI without oil[b]	1	10	0.4
Piezo-ICSI, sham operation[c]	2	10	0.95 (0.6–1.3)[e]
No injection[d]	2	10	0.35 (0.3–0.4)[e]

The drops (mHTF) of 100 μl were prepared in the chamber for measurement of mercury concentration.
[a]Fifty unfertilized mouse oocytes were injected with human spermatozoa in the same drop. This procedure was carried out in 10 drops per experiment.
[b]Performed without mineral oil between mercury and mHTF at the tip of injection pipette.
[c]Sham operation without oocytes and spermatozoa was performed.
[d]Mercury concentration of the drops was measured.
[e]Range of mercury concentration.

without deformation by vibrating the injection needle fixed on the piezodriver. Similarly, after the oolemma is extended by inserting the needle, the oolemma can be punctured by applying one piezo pulse. In mice, the plasma membrane has high extendibility and ooplasm has low viscosity. As a result, the secure injection of the spermatozoon into the oocyte was considered difficult, and Kimura and Yamagimachi (1995) reported an 80% survival rate and 78% fertilization rate to injected oocytes using their method. We applied their method to human ICSI, and observed a significant increase in survival and fertilization rates. Improvements in survival rate were, according to Kimura and Yamagimachi (1995), due to the fact that the extended oolemma agglutinates more securely after extraction of the needle, and the internal pressure of the ooplasm is lowered due to less deformation. In addition, oocytes are sufficiently activated by sperm factor brought in by an injected spermatozoon (Homa *et al.*, 1994; Tesarik *et al.*, 1994; Parrington *et al.*, 1996; Palermo *et al.*, 1997). Thus, no suction of ooplasm is necessary, which makes it possible to conduct ICSI while minimizing damage to oocytes. Improvements in the fertilization rate were considered due to the elimination of failures in the injection of spermatozoa, as spermatozoa were injected only after the puncture of oolemma by the needle was confirmed. An injection needle with a flat tip is used in piezo-ICSI. The mechanism of smooth puncture of the zona pellucida and oolemma by the needle seems to be as follows: the needle vibrates back and forth by taking piezo pulses. At this point, since inertia of mercury at the tip of the needle is high, rapid variation in internal pressure is caused in the lumen from mercury to the tip, and by pressing the flat tip of the needle against the zona pellucida, the zona is perforated in the shape of the tip lumen. Deep insertion of the needle into the ooplasm by hand causes no rupture of the oolemma in ~82% of metaphase II oocytes (unpublished data), but applying one piezo pulse to such oocytes causes rupture of the oolemma by variation in the internal pressure at the tip of the needle. It is at the time when a needle breaks the oolemma that vibration is transmitted to an oocyte. Because the vibration added is one piezo pulse and no suction of ooplasm is conducted at the sperm injection, it is thought that the damage caused by piezo-ICSI is much less than that by conventional ICSI. Grinding and spiking of the tip of the needle are not necessary in piezo-ICSI, which is considered an advantage because it reduces the amount of preparation required by researchers. In conventional ICSI, immobilization of motile spermatozoa to be injected is conducted by abrasion of sperm tails or pipetting, while in piezo-ICSI it can be conducted relatively easily by applying several piezo pulses to sperm tails. Huang *et al.* (1996) first reported the application of the piezo-manipulator to humans. In this method, an injection needle having a unbevelled tip was also used, and they concluded that the fertilization rate and pregnancy rate were comparable to those for other successful techniques. However, they did not report any results comparing piezo-ICSI with conventional ICSI in their hospital.

With regard to embryonic development after ICSI, since no suction of ooplasm is conducted in piezo-ICSI, it was presumed that the influence on the cytoskeleton would be slight and a higher rate of embryos with good morphology could be expected. However, no difference was recognized between the two groups by grade classification of the embryos after injection. On the other hand, pregnancy rates were improved by piezo-ICSI, which is considered due to the higher number of transferred oocytes per embryo transfer that were obtained from higher survival and fertilization rates in piezo-ICSI.

We applied piezo-ICSI to human ICSI. This method was the same as that reported by Kimura and Yanagimachi (1995) in mice. In their report, the oocytes fertilized by piezo-ICSI developed into blastocysts better than those by conventional ICSI (68 versus 33%). All eight foster mothers receiving sperm-injected oocytes (by piezo-ICSI) delivered their own and foster offspring, and all pups grew into normal adults. It is thought that the mercury contained in the injection needle does not influence development of the embryo. In our experiment, mercury (0.6 μg/l) was detected in the injection drop. The mercury concentration of blood in healthy humans was

reported to be 0.3–1.05 μg/l (Abraham *et al.*, 1984 ; Snapp *et al.*, 1989). In our data (unpublished), the mean concentration of mercury in the cervical mucus of women was 220 μg/l and the concentration in the seminal plasma of men was 52.4 μg/l. Therefore, the value of mercury concentration in the injection drop is thought to be comparatively low and safe.

In conclusion, stable and favourable fertilization results can be obtained by piezo-ICSI, which is a promising method for obtaining improved ICSI results.

Acknowledgement

The authors declare no vested interest of a commercial nature in this technique.

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