Performing ICSI Using an Injection Pipette with the Smallest Possible Inner Diameter and a Long Taper Increases Normal Fertilization Rate, Decreases Incidence of Degeneration and Trippronuclear Zygotes, and Enhances Embryo Development

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Purpose: To compare the efficacy of two types of injection pipette used for ICSI, one with a larger (5–7 μm) inner diameter and a shorter taper with that inner diameter, and another with the smallest (3–5 μm) possible inner diameter and a longer taper with that inner diameter.

Methods: Retrieved oocytes at metaphase II stage were injected using one of two types of injection pipette, in 33 and 94 cycles, respectively, in a total of 127 cycles in 108 patients.

Results: In comparison to the injection pipette with a larger (5–7 μm) inner diameter and a shorter taper with that inner diameter, the injection pipette with the smallest (3–5 μm) possible inner diameter and a longer taper with that inner diameter increased normal fertilization rate ($(70 \pm 3.6)\%$ vs. $(86 \pm 2.2)\%$; $P = .001$; mean $\pm$ SEM); decreased the incidence of degeneration ($(14 \pm 2.4)\%$ vs. $(5 \pm 1.4)\%$; $P = .001$) and trippronuclear zygotes ($(1.0 \pm 0.35)\%$ vs. $(0.1 \pm 0.21)\%$; $P = .03$); increased Day-2 diploid embryos ($(69 \pm 3.7)\%$ vs. $(85 \pm 2.2)\%$; $P = .001$) and good-quality Day-2 diploid embryos ($(67 \pm 4.0)\%$ vs. $(79 \pm 2.4)\%$; $P = .03$), all per injected oocyte; and increased the number of blastomeres per good-quality Day-2 diploid embryo ($(3.0 \pm 0.21$ vs. 3.8 ± 0.12 ; $P = .0003$).

Conclusions: Performing ICSI using an injection pipette with the smallest (3–5 μm) possible inner diameter and a longer taper with that inner diameter maximizes normal fertilization rate, minimizes the incidence of postinjection de-

generation and trippronuclear zygotes, and enhances embryo development.

KEY WORDS: degeneration; embryo; fertilization; ICSI; oocyte.

INTRODUCTION

Following the first pregnancy resulting from transfer of ICSI-produced embryos (1), ICSI has been the most powerful and widely used tool to treat couples with infertility due to severe oligozoospermia and/or asthenozoospermia, as well as to treat couples who have failed to achieve fertilization with conventional IVF despite normal sperm parameters. Although the fertilization rate from ICSI was initially as low as 40% per injected mature oocyte, it has gradually increased over the years, despite variations among centers and even among operators within centers, to 75–80% per injected oocyte. This has been mainly due to improvements over the years in the ICSI technique such as damaging the sperm tail membrane before its injection for post-ICSI sperm nucleus decondensation (2–7) and for the release of the spermatozoon-associated oocyte-activating factor (8–10); and aspiration of ooplasm before the injection of the spermatozoon for oolemma rupture (7,11–13) and for oocyte activation (14,15); as well as improvements in micromanipulation instruments and in operators' skills. As a result of this improvement in its efficiency, the ICSI technique has recently offered even higher fertilization rates than the conventional IVF for non-male-factor infertility, when the fertilization rate is based on the number of sibling oocytes

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allocated to ICSI rather than on the number of injected oocytes (65.6% vs. 52.5%, 16; 71.3% vs. 57.2%, 17), whereas this difference was not observed in previous reports (63% vs. 50.7%, 18; 60.4% vs. 54%, 19), and in a recent report (62% vs. 53%, 20).

At 15–18 h after injection of a mature (MII) oocyte, the injected oocyte either displays 2 pronuclei (2PN; normal fertilization), degeneration, no PN (intact oocyte surviving ICSI), 1PN (parthenogenetic activation), or 3PN (abnormal fertilization). Postinjection degeneration is a common problem seen after ICSI, ranging from 4 to 17%, whereas incidence of 1PN and 3PN ranges from 0 to 11% and 0 to 7%, respectively. Since all the MII oocytes hypothetically have the potential to fertilize when injected with a normal spermatozoon, the potential 100% normal fertilization rate after ICSI is compromised mainly by the considerably high incidence of post-ICSI degeneration and, to a lesser degree, by the formation of mono- and trippronuclear zygotes. Although degeneration of the injected oocytes, and fertilization failure of the injected and surviving intact oocytes, can be minimized by improved expertise and skills of the operator, they are still inevitable even in the hands of the most experienced operators. Considering the fact that the ICSI fertilization rate gradually increased over the years and considering the relatively-high incidence of degeneration of the injected oocytes, there is always a room to maximize the ICSI fertilization rate. Thus, one way to maximize normal fertilization rate from ICSI would be to minimize the degeneration and abnormal fertilization of the injected oocytes.

In February 1999, the manufacturer of our micropipettes modified their injection pipettes by decreasing the inner diameter of the pipette from 5–7 μm to 3–5 μm , and by increasing the length of the

taper with that inner diameter, thus manufacturing injection pipettes with a flexible (nonrigid) taper. As soon as we started using this new type of injection pipettes, we noticed a sudden decrease in the post-ICSI degeneration rate and an increase in normal fertilization rate of injected oocytes in our program. Considering that the design of the injection pipette may indeed contribute to the ICSI outcome, we retrospectively compared the efficacy of the two types of injection pipette by analyzing the incidence of degeneration, normal fertilization, formation of mono- and trippronuclear zygotes, and embryo development.

MATERIALS AND METHODS

Patients

One-hundred and eight women who had enrolled in our IVF program were assigned either to full ICSI (78 cycles) or partial ICSI (49 cycles), in a total of 127 cycles (Table I). In patients assigned to partial ICSI, randomly half of the retrieved oocyte–cumulus complexes in a cycle were assigned to ICSI. Female partners' ages ranged from 21 to 47 years with an average of 35 ± 5.2 (mean $\pm$ SD) and a median of 35 years. The length of infertility ranged from 0.5 to 18 years, with an average of 5.9 ± 4.39 years. The cause of infertility was diagnosed to be tubal ($n = 44$), male-related ($n = 32$), male-related and tubal ($n = 8$), endometriosis ($n = 4$), unknown ($n = 38$), and one woman had oocyte donation. All couples were interviewed by a counsellor (M.W.E.) and were provided with a detailed informative explanation of the procedure. The treatments were carried out between August 1998 and November 1999.

Table I. Infertility Characteristics of the Female Partners and Semen Characteristics of the Male Partners in 108 Couples, Whose Oocytes and Sperm, Respectively, Were Subjected to ICSI Using One of Two Types of Injection Pipette

Variable	Type of injection pipette		<i>P</i>
	Larger (5–7 μm) inner diameter, shorter taper	Smallest (3–5 μm) inner diameter, longer taper	
No. of cycles	33	94	—
No. of full ICSI cycles	28	50	—
No. of partial ICSI cycles	5	44	—
Age (year) ^a	35 ± 0.9	35 ± 0.5	.47
Length of infertility (year) ^a	6.8 ± 2.21	5.8 ± 0.68	.69
Semen volume (mL) ^a	3.7 ± 0.31	3.0 ± 0.19	.06
Sperm concentration ($\times 10^6/\text{mL}$) ^a	34 ± 6.6	56 ± 3.9	.004
Sperm forward-progressive motility (%) ^a	41 ± 3.1	41 ± 1.9	.98

^a Mean $\pm$ SEM.

Controlled Ovarian Stimulation and Oocyte Retrieval

A standard ovarian suppression protocol was utilized with Buserelin treatment from Day 20 of the menstrual cycle for 10 days or more until the serum estradiol concentration declined to less than 184 pmol/L. The patients then received 3–6 ampoules of hMG (75 IU) per day until at least two follicles reached a diameter of 1.8 cm. At this time, hCG (10,000 IU; Profasi, Serono) was given im in the evening, and oocyte retrieval was performed 35 h later as described previously (21).

Oocyte Culture

The retrieved oocyte–cumulus complexes were washed 2–3 times in human tubal fluid (HTF) culture medium, transferred individually into 100- μ L droplets of culture medium containing HSA (IVF Science Scandinavia, Gothenburg, Sweden) under paraffin oil (Bell, Sons & Co., Southport, England), and then put in an incubator (37°C, 5% CO₂). Approximately 4–5 h after retrieval, the oocytes were stripped of the surrounding cumulus cells: the oocyte–cumulus complexes were placed for 45 s in 0.01% (wt/vol) hyaluronidase (Sigma, St. Louis, MO) in Earle's balanced salt solution (Gibco BRL Life Technologies, Grand Island, NY), containing 10% HSA (Bayer), rinsed twice in Earle's solution, and then aspirated in and out of pipettes of 150–200 μ m inner diameter. The stripped oocytes were then transferred into fresh drops and assessed for maturity; oocytes that were at metaphase II stage (MII oocytes), i.e., oocytes with the first polar body extruded, were defined as mature. Only oocytes at metaphase II stage were injected with a spermatozoon.

Sperm Processing

After completion of liquefaction, semen specimens were washed by the gradient density method using the Enhance-S Plus solution (Conception Technologies, San Diego, CA), and the resulting pellet was used for ICSI. Severely oligozoospermic semen samples (<10⁶ motile sperm/mL) were centrifuged for 5–10 min at 300 g, and the resulting pellet was used for ICSI.

Description of Micropipettes

Both holding pipettes (catalog# HP-95-15) and injection pipettes (catalog# IC-15) were manufactured

by the same company (Conception Technologies, San Diego, CA). Holding pipettes had an inner diameter of 95 μ m, an opening of 15–25 μ m, and a 15° bend at 1 mm from the tip. Injection pipettes used during the first phase of the study (August 1998 to February 1999; larger-diameter injection pipettes in 33 cycles) had an inner diameter of 5–7 μ m, and a shorter (rigid) taper with that inner diameter. Injection pipettes used during the second phase of the study (February 1999 to November 1999; smallest-diameter injection pipettes in 94 cycles) had an inner diameter of 3–5 μ m, and a longer (nonrigid, flexible) taper with that inner diameter. Both types of injection pipette had a 15° bend at 1 mm from the tip, and a bevel of 18–25° at the tip.

The injection pipettes had originally been made from borosilicate glass tubing with an outer diameter of 1,000 μ m and an inner diameter of 750 μ m. In order to calculate the approximate volumes within the injection pipette at various distances from the tip of the pipette, both types of injection pipette were hypothetically divided into sections at 0.5 mm, 1 mm, and thereafter at 1-mm intervals until 10 mm from the tip, and the inner diameter was measured at these specified intervals using a grid micrometer attached to the eyepiece of the microscope (Table II). Hypothetical division of injection pipettes resulted either in cylindrical sections (between 6 and 10 mm of the larger-diameter injection pipette, and between the tip and 1 mm, and between 9 and 10 mm of the smallest-diameter injection pipette) or in conical sections (at the remaining intervals of both types of injection pipette). The volumes of the cylindrical sections were calculated using the following formula (22):

$$V = r^2\pi h,$$

where V is the volume of the cylindrical section, r is the radius of the cylindrical section, and h is the length (height) of the cylindrical section (0.5 or 1 mm). The volumes of the conical sections were calculated by the trapezoidal numerical integration method using the following formula (22):

$$V = [(r_1 + r_2)/2]^2\pi h,$$

where V is the volume of the conical section, r_1 is the smaller radius of the conical section (closer to the tip), r_2 is the larger radius of the conical section (away from the tip), and h is the length (height) of the conical section (0.5 or 1 mm). The cumulative volumes within the injection pipette from the tip to each of these intervals were then determined for each type of injection pipette (Table II).

Table II. The Inner Diameters, Section Volumes, and Cumulative Volumes at Different Intervals from the Tip of Injection Pipettes with Either a Larger (5–7 μm) Inner Diameter and a Shorter Taper, or the Smallest (3–5 μm) Inner Diameter and a Longer Taper

Distance from tip of pipette (mm)	Inner diameter (μm)		Section volume ^a ($10^6 \mu\text{m}^3$)		Cumulative volume ^b ($10^6 \mu\text{m}^3$)		V1/V2
	Larger-diameter pipette	Smallest-diameter pipette	Larger-diameter pipette	Smallest-diameter pipette	Larger-diameter pipette (V1)	Smallest-diameter pipette (V2)	
0	6	4	—	—	—	—	—
0.5	10	4	0.025	0.006	0.025	0.006	4.2
1	15	4	0.061	0.006	0.086	0.013	6.6
2	35	25	0.490	0.165	0.577	0.178	3.2
3	150	50	6.7	1.1	7.3	1.3	5.6
4	300	90	40	3.8	47	5.1	9.2
5	500	140	126	10	173	16	10.8
6	750	270	307	33	480	49	9.8
7	750	370	442	80	921	129	7.1
8	750	480	442	142	1,363	271	5.0
9	750	750	442	297	1,805	568	3.2
10	750	750	442	442	2,247	1,010	2.2

^a Section volume for a given distance from tip of pipette is the volume of the cylindrical or the conical section between that distance and the preceding distance.

^b Cumulative volume for a given distance from tip of pipette is the sum of the section volume for that distance and the cumulative volume for the preceding distance.

ICSI Technique

The ICSI procedure was performed by the same scientist (Y.Y.) throughout the study period. The micromanipulation equipment consisted of a microinjector with oil pressure system and a microholder (Narishige, Japan), which were fitted to an inverted microscope (Nikon, Japan). Several 20- μL drops of 10% polyvinylpyrrolidone (PVP; Sigma, St. Louis, MO) and several 20- μL drops of Earle's solution with 10% HSA were placed in the lid of a Falcon petri dish (100 $\times$ 15 mm; Fisher Scientific, Pittsburgh, PA), overlaid by paraffin oil. Five microliters of washed sperm or sperm pellet was placed in the centers of PVP drops, and one oocyte in each drop of Earle's solution. Under the microscope, a motile spermatozoon with normal morphology was selected in the bottom of the dish lid, and made immotile by squeezing its upper-tail region between the tip of the injection pipette and the bottom of the dish lid, and with the backward movement of the tip of the injection pipette. The immobilized spermatozoon was then aspirated tail first into the injection pipette. The oocyte was held by the holding pipette with its first polar body positioned either at the 6 or 12 o'clock position. The spermatozoon was brought as close to the tip of the injection pipette as possible. The injection pipette was firmly pushed into the ooplasm at the 3 o'clock position until past half-way inside the ooplasm when the oolemma surrounding the injection pipette recoiled. At this time, the injection pipette was moved inside the ooplasm as close to the

oolemma at the 9 o'clock position as possible, and the ooplasm with the corresponding oolemma at the 3 o'clock position was aspirated into the injection pipette until a rapid and free flow of ooplasm was observed in the injection pipette as a sign of oolemma rupture. The spermatozoon was then slowly released into the ooplasm along with minimal amount of PVP. The injection pipette was withdrawn gently from the oocyte; the oocyte was released from the holding pipette, rinsed thoroughly, immediately transferred into fresh culture drops, and returned to the incubator. A maximum of four oocytes were placed in the dish lid at a time so that they could be injected and returned to the incubator within 15 min.

Assessment of Fertilization and Embryo Development

At 15–18 h after ICSI (Day 1; Day 0 = day of oocyte retrieval), the injected oocytes were observed under microscope for the presence of 2PN, 3PN, 1PN, 0PN, and degeneration. At 48 h postretrieval (Day 2), the zygotes that had displayed 2PN were assessed for cleavage, and those that had cleaved (Day-2 diploid embryos) were graded as good-quality embryos (equal-size blastomeres with fragmentation $\leq 20\%$ of the size of the embryo) or poor-quality embryos (unequal-size blastomeres and/or blastomeres with fragmentation $> 20\%$ of the size of the embryo). The oocytes that had displayed 3PN, 1PN or 0PN, or that had degenerated on Day 1 were not assessed any further after Day 1.

Statistical Analyses

Female partner's age and length of infertility, and the male partner's semen volume, sperm concentration, and sperm forward-progressive motility were compared between the two types of injection pipette by analysis of variance, using the General Linear Models Procedure of SAS (23). The following variables were compared between the two types of injection pipette by analysis of covariance with the female partner's age and the male partner's sperm concentration and sperm forward-progressive motility as covariates, using the General Linear Models Procedure of SAS (23): proportions of injected oocytes that degenerated, and that displayed 2PN, 3PN, 1PN, and 0PN; proportions of Day-2 diploid embryos per 2PN-zygote and per injected oocyte; proportions of good-quality Day-2 diploid embryos per Day-2 diploid embryo, per 2PN-zygote, and per injected oocyte; and number of blastomeres per good-quality Day-2 diploid embryo. Correlation coefficients among female partner's age, male partner's sperm concentration and sperm forward-progressive motility, and ICSI outcome variables were determined within the two types of injection pipette, using the Correlation Procedure of SAS (23). Differences were assumed to be significant at $P < .05$.

RESULTS

Infertility characteristics of the female partners and semen characteristics of the male partners are shown in Table I. With the exception of the sperm concentration, these variables did not differ between the two groups of patients who had ICSI using two different types of injection pipette.

Day-1 fertilization status of injected oocytes is shown in Table III. Performing ICSI using an injection

pipette with the smallest (3–5 μm) possible inner diameter and a longer taper with that inner diameter, in comparison to an injection pipette with a larger (5–7 μm) inner diameter and a shorter taper with that inner diameter, decreased degeneration rate ($P = .001$), increased normal fertilization (2PN) rate ($P = .001$), and decreased the abnormal fertilization (3PN) rate ($P = .03$; Table III). The decrease in parthenogenetic activation (1PN; $P = .65$) and in fertilization failure of the surviving intact oocyte (0PN with no degeneration; $P = .37$) was not significant (Table III). With the use of the larger-diameter injection pipette, 1 of 33 cycles (3%; 3 oocytes injected) resulted in total fertilization failure, whereas with the use of the smallest-diameter injection pipette, only 1 of 94 cycles (1%; 1 oocyte injected) resulted in total fertilization failure ($P = .44$; Table III).

Day-2 embryo development status of 2-PN zygotes is shown in Table IV. Performing ICSI using the smallest-diameter injection pipette, in comparison to the larger-diameter injection pipette, resulted in increased Day-2 diploid embryos per injected oocyte ($P = .001$), but not per 2PN-zygote ($P = .38$); in increased good-quality Day-2 diploid embryos per injected oocyte ($P = .03$), but not per 2PN-zygote ($P = .24$) or per Day-2 diploid embryo ($P = .10$); and in increased number of blastomeres per good-quality Day-2 diploid embryo ($P = .0003$; Table IV).

The female partner's age as well as the male partner's sperm concentration and the sperm forward-progressive motility included as covariates in the statistical model did not affect any of the ICSI outcome variables ($.06 < P < .98$). Female partner's age, male partner's sperm concentration, and male partner's sperm forward-progressive motility were not correlated ($.06 < P < .98$) with the ICSI outcome variables.

Table III. Day-1 Fertilization Status of Injected Oocytes After ICSI Using One of Two Types of Injection Pipette in 108 Women

Variable	Type of injection pipette		<i>P</i>
	Larger (5–7 μm) inner diameter, shorter taper	Smallest (3–5 μm) inner diameter, longer taper	
No. of cycles	33	94	—
No. of oocytes injected	226	475	—
Cycles with total fertilization failure (%)	3 (1/33)	1 (1/94)	.44
Degeneration rate (%) ^a	14 $\pm$ 2.4	5 $\pm$ 1.4	.001
2PN (%) ^a	70 $\pm$ 3.6	86 $\pm$ 2.2	.001
3PN (%) ^a	1.0 $\pm$ 0.35	0.1 $\pm$ 0.21	.03
1PN (%) ^a	2.3 $\pm$ 0.87	1.4 $\pm$ 0.51	.65
0PN (%) ^a	12 $\pm$ 2.8	8 $\pm$ 1.6	.37

^a Mean $\pm$ SEM per injected oocyte.

Table IV. Day-2 Embryo Development Status of 2PN-Zygotes Resulting from ICSI Using One of Two Types of Injection Pipette in 108 Women

Variable	Type of injection pipette		P
	Larger (5–7 μ m) inner diameter, shorter taper	Smallest (3–5 μ m) inner diameter, longer taper	
No. of cycles	33	94	—
Day-2 diploid embryos			
Per 2PN-zygote (%) ^a	98 $\pm$ 0.9	99 $\pm$ 0.6	.38
Per injected oocyte (%) ^a	69 $\pm$ 3.7	85 $\pm$ 2.2	.001
Good-quality Day-2 diploid embryos			
Per Day-2 diploid embryo (%) ^a	97 $\pm$ 2.3	93 $\pm$ 1.3	.10
Per 2PN-zygote (%) ^a	95 $\pm$ 2.4	93 $\pm$ 1.4	.24
Per injected oocyte (%) ^a	67 $\pm$ 4.0	79 $\pm$ 2.4	.03
No. of blastomeres per good-quality Day-2 diploid embryo ^a	3.0 $\pm$ 0.21	3.8 $\pm$ 0.12	.0003

^a Mean $\pm$ SEM.

DISCUSSION

Normal Fertilization

Switching from an injection pipette with a larger (5–7 μ m) inner diameter and a shorter taper with that inner diameter to an injection pipette with the smallest (3–5 μ m) possible inner diameter and a longer taper with that inner diameter significantly increased normal fertilization rate of injected oocytes in the present study. This improvement was not due to an improvement in the operator's skills with time because the normal fertilization rate with the larger-diameter injection pipette (70%) was already in the upper range of that reported in the literature (56–78%). This difference was also not due to variation in different ICSI operators because all ICSI procedures throughout the study were performed by the same scientist. Furthermore, there was no variation due to different manufacturers of micropipettes since all micropipettes used throughout the study were manufactured by the same company.

The female partner's age and the male partner's sperm forward-progressive motility were not different between the two groups of patients. Although the male partner's sperm concentration was lower in the group where ICSI was performed using the larger-diameter injection pipette than in the group where ICSI was performed using the smallest-diameter injection pipette, it was still higher than that required for a normal semen specimen (20×10^6 /mL) by World Health Organization (24). Furthermore, analysis of the data showed that the normal fertilization rate from ICSI was not affected by the female partner's age and the male partner's sperm parameters, confirming the previous reports (25,26).

Fertilization failure of the surviving intact oocyte (0PN with no degeneration) is either due to 1) failure to aspirate enough ooplasm into the injection pipette prior to injection to induce oolemma rupture (7,11–13), to prevent the post-ICSI ejection of the spermatozoon (27,28), and to induce oocyte activation (2,14,15,27–29); or 2) failure to damage the tail membrane of the spermatozoon to induce sperm nucleus decondensation (2,3,27,28), to release the spermatozoon-associated oocyte-activating factor (8–10), and to increase the normal fertilization rate (4–7,30). In the present study, the significant increase in the normal fertilization rate after changing the pipette type was not associated with a significant decrease in the incidence of fertilization failure of the surviving intact oocyte (0PN with no degeneration), but it was associated with a significant decrease in the degeneration rate and the incidence of 3PN. This indicates that the increase in the normal fertilization rate was in fact a result of a decrease in the degeneration rate and, to lesser degree, a result of a decrease in the incidence of 3PN, rather than an actual increase in the normal fertilization rate per se.

Incidence of Degeneration

The significant decrease in the degeneration rate after changing the type of the injection pipette was not due to an improvement in the operator's skills with time because the degeneration rate with the larger-diameter injection pipette (14%) was already within the range of that reported in the literature (4–17%). Furthermore, the degeneration rate after ICSI was not affected by the female partner's age and the male partner's sperm parameters. Various mechanisms have been suggested for the occurrence of

post-ICSI degeneration of injected oocytes: 1) failure of the oolemma to heal its rupture following withdrawal of the injection pipette, resulting in increased chance of the ooplasm coming into contact with the extracellular environment; 2) excessive amount of ooplasm aspirated into the injection pipette prior to injection, resulting in the damage to intracellular components, especially to the metaphase II spindle; and 3) excessive amount of PVP injected into the oocyte along with the spermatozoon.

Failure of the oolemma to heal its rupture following withdrawal of the injection pipette is determined by the size of the breach in the oolemma made by the injection pipette. It was suggested that ooplasmic aspiration is an invasive part of ICSI (7), that degeneration is caused by the mechanical trauma to the oolemma during injection (31), and that an injection pipette with the smallest possible inner diameter be used for ICSI (7). In the second phase of the present study, the injection pipette had indeed the smallest (3–5 μm) possible inner diameter as the width of the head of a normal spermatozoon ranges from 2.5 to 3.5 μm (24), and performing ICSI with this type of injection pipette minimized the incidence of degeneration. Assuming that the outer diameter of an injection pipette is 1 μm greater than its inner diameter, the outer diameter of the larger-diameter (5–7 μm) injection pipette and of the smallest-diameter (3–5 μm) injection pipette would be 7 μm ($r = 3.5 \mu\text{m}$) and 5 μm ($r = 2.5 \mu\text{m}$), respectively. Applying the formula for the area of a circle ($A = r^2\pi$), the size of the breach made in the oolemma would be $3.5^2 \times \pi = 12.25\pi \mu\text{m}^2$ for the larger-diameter injection pipette, and $2.5^2 \times \pi = 6.25\pi \mu\text{m}^2$ for the smallest-diameter injection pipette. Thus, the breach made in the oolemma by the smallest-diameter injection pipette was almost half the size of that made by the larger-diameter injection pipette. Furthermore, a conventional intracellular electrode with a diameter smaller than that of an injection pipette resulted in a higher transmembrane electric potential on the oolemma than did the injection pipette, suggesting that the smaller the diameter of the injection pipette the easier the penetration of the oolemma (32). Thus, using an injection pipette with the smallest possible inner diameter in the present study may also have facilitated the penetration of the oolemma, resulting in higher fertilization rate.

During the ICSI procedure, the oocyte is held by the holding pipette with its first polar body positioned

either at the 6 or 12 o'clock position to ensure that the injection pipette pushed into the oocyte at the 3 o'clock position stays away from the metaphase II spindle. However, the first polar body moves in the perivitelline space during the denuding procedure prior to ICSI (33,34). Thus, it is believed that ooplasmic aspiration into the injection pipette prior to injection may cause degeneration in some of the injected oocytes by damaging the metaphase II spindle which is not located near the first polar body (35). One way to avoid the damage to the metaphase II spindle may be not to aspirate at all the ooplasm into the injection pipette prior to injection. It was reported (36) that when the ooplasm was not aspirated into the injection pipette prior to injection, the incidence of degeneration decreased from 16.8 (associated with ooplasmic aspiration) to 4.6%, with no change in normal fertilization rate (61.4% vs. 62.5% respectively). However, ooplasmic aspiration into the injection pipette prior to injection is necessary for oocyte activation (14,15) and oolemma rupture (7,12,13), and thereby to increase the normal fertilization rate (7,11,12,15).

An alternative way to minimize the damage to the metaphase II spindle would be to minimize the amount of ooplasm aspirated into the injection pipette prior to injection. It was suggested that the amount of ooplasm aspirated into the injection pipette prior to injection be reduced by using an injection pipette with the smallest possible inner diameter (7). In the second phase of the present study, the injection pipette had the smallest (3–5 μm) possible inner diameter and a long taper (1 mm) with that inner diameter, and performing ICSI with this type of injection pipette minimized the incidence of degeneration. When an oocyte is positioned at the far left edge of the microscopic view at 400 $\times$ magnification (using the 40 $\times$ objective) during ICSI, its ooplasm has to be aspirated along with the corresponding intact oolemma into the injection pipette at least until the far right edge of the microscopic view where the oolemma rupture is visualized as evidenced by a rapid and free flow of the ooplasm. Since the diameter of the microscopic view at 400 $\times$ magnification corresponds to about 0.5 mm, one can assume that, for oolemma rupture prior to injection, the volume of ooplasm aspirated into the larger-diameter injection pipette until 0.5 mm from the tip was about $0.025 \times 10^6 \mu\text{m}^3$, and the volume of ooplasm aspirated into the smallest-diameter injection pipette until 0.5 mm from the tip was only about $0.006 \times 10^6 \mu\text{m}^3$ (Table II). Thus, for oolemma rupture before the injection, the

amount of ooplasm that had to be aspirated into the smallest-diameter injection pipette was about one fourth the amount of ooplasm that had to be aspirated into the larger-diameter injection pipette. Furthermore, since an MII oocyte has an average diameter of $110\text{ }\mu\text{m}$ ($r = 55\text{ }\mu\text{m}$) excluding the perivitelline space and the zona pellucida (37), the total volume of the ooplasm is $(4/3)r^3\pi = (4/3) \times 55^3 \times \pi = 696,910\text{ }\mu\text{m}^3 \approx 0.7 \times 10^6\text{ }\mu\text{m}^3$. The volume of ooplasm aspirated into the larger-diameter injection pipette ($0.025 \times 10^6\text{ }\mu\text{m}^3$; Table II) corresponds to 3.6% ($0.7 \times 10^6\text{ }\mu\text{m}^3$) of the total ooplasm volume, whereas the volume of ooplasm aspirated into the smallest-diameter injection pipette ($0.006 \times 10^6\text{ }\mu\text{m}^3$; Table II) corresponds to only 0.9% ($0.7 \times 10^6\text{ }\mu\text{m}^3$) of the total ooplasm volume. Thus, performing ICSI using the smallest-diameter injection pipette reduced the amount of ooplasm that had to be aspirated for oolemma rupture, and the associated risk of damaging the metaphase II spindle, to one fourth of that associated with the use of the larger-diameter injection pipette. Indeed, using the smallest-diameter injection pipette decreased the degeneration rate.

The PVP is used to reduce sperm motility for facilitating aspiration of a motile spermatozoon, and to coat inside the injection pipette for preventing an aspirated spermatozoon from sticking to the inner wall of the injection pipette. As a result, some PVP is inevitably injected along with the spermatozoon into the oocyte during ICSI. The PVP may interfere with sperm nucleus decondensation (3), be detrimental to the ultrastructural components of spermatozoa (38), and be potentially mutagenic (39). Thus, it is believed that excessive amount of PVP injected along with the spermatozoon into the oocyte during ICSI may cause degeneration in some of the injected oocytes (35). Although acceptable ICSI fertilization rates have been reported without the use of PVP (40–43), these techniques are not very practical, and require extra extensive training of embryologists (41). An alternative way to minimize the incidence of degeneration caused by PVP would be to minimize the amount of PVP injected into the oocyte along with the spermatozoon. This again can be achieved by using an injection pipette with the smallest possible inner diameter and a longer taper with that inner diameter. In the second phase of the present study, the injection pipette had the smallest ($3\text{--}5\text{ }\mu\text{m}$) possible inner diameter and a long taper (1 mm) with that inner diameter, and performing ICSI with this type of injection pipette minimized the incidence of degeneration.

Incidence of 1PN and 3PN

In the present study, the incidences of 1PN (2.3%) and 3PN (1%) using the larger-diameter injection pipette were already in the lower range of those reported in the literature (0–11% and 0–7%, respectively). Switching to the smallest-diameter injection pipette further decreased the incidence of 1PN to 1.4%, although not significantly, and further and significantly decreased the incidence of 3PN to 0.1%. The incidence of 1PN and 3PN was not affected by the female partner's age and the male partner's sperm parameters. Formation of 3PN after ICSI is not due to dispermic fertilization (44), but to failure of the injected oocyte to extrude its second polar body after being injected (27,28,45). The further significant decrease in the incidence of 3PN after changing the type of the injection pipette in the present study could be because the amount of ooplasm that had to be aspirated into the smallest-diameter injection pipette for oolemma rupture was about one fourth the amount of ooplasm that had to be aspirated into the larger-diameter injection pipette, which may have resulted in reduced risk of damage to intracellular components, and facilitated the extrusion of the second polar body.

Embryo Development

It was suggested that, since ooplasmic aspiration is an invasive part of ICSI and may influence the quality of the embryos, the amount of ooplasm aspirated into the injection pipette prior to injection be reduced by using an injection pipette with the smallest possible inner diameter (7). The fact that the difference in the embryo quality between the two types of injection pipette was observed per injected oocyte, but not per Day-2 diploid embryo or per normally fertilized oocyte (2PN-zygote), indicates that the improvement in the embryo quality was a result of better fertilization and, thus, it was related to the type of the injection pipette, and not to the postinjection period. The fact that the good-quality embryos were defined as embryos with equal-size blastomeres with fragmentation $\leq 20\%$ of the size of the embryo indicates that switching to the smallest-diameter injection pipette reduced the degree of embryo fragmentation as a result of better fertilization. This reduction in fragmentation was due most likely to the fact that the amount of ooplasm that had to be aspirated into the smallest-diameter injection pipette for oolemma rupture was about one fourth the amount of ooplasm that

had to be aspirated into the larger-diameter injection pipette, resulting in reduced damage to intracellular components. Switching to the smallest-diameter injection pipette also significantly increased the number of blastomeres per good-quality Day-2 diploid embryo from 3.0 to 3.8, which indicates that performing ICSI with the smallest-diameter injection pipette was followed by a faster cleavage of normally fertilized oocytes.

It was reported that ooplasmic aspiration into the injection pipette prior to injection increased the incidence of degeneration from 4.6 to 16.8%, and decreased the good-quality embryos from 48.5 to 24.5% compared with no ooplasmic aspiration, with fertilization rate unaffected (62.5% vs. 61.4%, respectively), all per injected oocyte (36). In contrast, we have shown that, with ooplasmic aspiration but into the smallest-diameter injection pipette, it is possible to minimize the incidence of degeneration to 5%, maximize the fertilization rate to 86%, and maximize the good-quality embryos to 79%, all per injected oocyte. Thus, our results again suggest that minimizing the amount of ooplasm aspirated into the injection pipette prior to injection is not only a highly efficient alternative method to no ooplasmic aspiration at all aimed at minimizing degeneration, but also a method to maximize normal fertilization rate and enhance embryo development.

CONCLUSIONS

Performing ICSI with an injection pipette with the smallest (3–5 μm) possible inner diameter and a longer taper with that inner diameter, in comparison to an injection pipette with a larger (5–7 μm) inner diameter and a shorter taper with that inner diameter, decreased the incidence of postinjection degeneration and trippronuclear zygotes, which resulted in higher normal fertilization rate and better embryo development with reduced fragmentation and faster cleavage. The lower incidence of degeneration from ICSI using the smallest-diameter injection pipette was likely a result of minimized degree of the mechanical trauma exerted on the oolemma, minimized amount of ooplasm aspirated into the injection pipette prior to injection, and minimized amount of PVP injected into the oocyte along with the spermatozoon. The results of the present study suggest that particular attention be paid to the design of the injection pipettes to maximize the outcome from ICSI.

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