

ORIGINAL ARTICLE

Piezo-assisted ICSI improves fertilization and blastocyst development rates compared with conventional ICSI in women aged more than 35 years

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Abstract

Purpose: Piezo-assisted intracytoplasmic sperm injection (Piezo-ICSI) is reported to be an effective method for inseminating fragile oocytes compared with conventional ICSI (c-ICSI). However, infertile patient groups suitable for Piezo-ICSI have not been elucidated. This study was conducted to determine age groups suitable for Piezo-ICSI using sibling egg controls inseminated by a well-trained embryologist to reduce technical inequalities.

Methods: A total of 947 matured oocytes were inseminated either by Piezo-ICSI or by c-ICSI in sibling oocytes as controls. Fertilization (2 pronuclei, PN), survival, and blastocyst development rates on day (D) 5 and D6 after insemination were compared between the Piezo-ICSI and c-ICSI groups. Further analyses were applied to groups of women >35 or ≤35 years of age.

Results: There were no significant differences in fertilization, survival, or blastocyst development rates between the two insemination treatment groups. However, for women >35 years of age, the fertilization ($P = .008$) and blastocyst development ($P = .016$) rates with Piezo-ICSI on D5 and D6 were significantly higher than in those subjected to c-ICSI.

Conclusions: Piezo-ICSI was useful for inseminating oocytes from women >35 years of age.

KEYWORDS

aging, blastocyst development rate, fertilization rate, intracytoplasmic sperm injection, Piezo-ICSI

1 | INTRODUCTION

Although intracytoplasmic sperm injection (ICSI) has been largely restricted to patients showing repeated failure of in vitro

fertilization (IVF), ICSI is widely recognized as a major technique in assisted reproductive technology (ART).¹ When conventional ICSI (c-ICSI) is performed, the injection needle penetrates the zona pellucida and then perforates the oolemma. Oocyte cytoplasm is

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then aspirated into the injection needle to rupture the oolemma, followed by injection of a spermatozoon. The injection process of c-ICSI may affect fertilization, blastocyst development, and pregnancy rates, as needle penetration of the plasma membrane and the aspiration of cytoplasm by negative pressure can have a negative impact on oocytes, particularly oocytes from older women because their oocytes have fragile plasma membranes. In fact, cell membrane viscosity and elasticity are known to change with age in several cell types, such as erythrocytes, lymphocytes, and red blood cells.² Piezo-assisted ICSI (Piezo-ICSI) markedly decreased physical pressure on the oocyte plasma membrane via a high-speed micro-advancing needle system. Kimura and Yanagimachi³ reported that Piezo-ICSI improved the survival rate (up to 80%) of mouse oocytes, with a 78% fertilization rate. Yanagida *et al* applied Piezo-ICSI to human oocytes and obtained improved survival (89% vs 81%) and fertilization (70% vs 54%) rates compared with c-ICSI.⁴ To date, there have been a limited number of reports on the application of Piezo-ICSI to human oocytes, from Huang's first report to a recent study by Hiraoka describing a very thin flattened pipette which may benefit Piezo-ICSI. Although Piezo-ICSI is expected to improve outcomes from the more fragile oocytes of older compared with younger women, limited information has been reported comparing outcomes from oocytes of aged and younger women by c-ICSI or Piezo-ICSI. We performed a prospective study using a well-trained embryologist to determine whether Piezo-ICSI may provide better outcomes than c-ICSI or be a more effective ICSI technique for oocytes from aged compared with younger women.

2 | MATERIALS AND METHODS

2.1 | Study population

From October to December 2017, 137 patients with 1356 oocytes were studied at Hanabusa Women's Clinic (Table 1). Patients were stimulated with a standard superovulation protocol (see below), and oocytes from cycles with more than two retrieved mature oocytes were used in this study. As a result, 947 mature oocytes were studied: Half of the oocytes from each patient were inseminated using c-ICSI and the remaining sibling oocytes were subjected to Piezo-ICSI. This study was approved by the Institutional Review Board of Hanabusa Women's Clinic (IRB number Hanabusa Women's Clinic #07).

2.2 | Oocyte retrieval, sperm preparation, and culture conditions

All patients were stimulated to super-ovulate with a standard long protocol, a short protocol, or a gonadotropin-releasing hormone (GnRH) agonist protocol, and clomiphene citrate or letrozole was administered to patients who showed a poor response to these standard protocols. Details of patients, anti-Müllerian hormone (AMH), basal follicle-stimulating hormone (FSH), and ovarian stimulation methods are shown in Table 1.

TABLE 1 Background of patients and ovarian stimulation protocols used before oocyte retrieval

Characteristics	Value
No. of patients	137
Age at ICSI (y) ^a	37.1 ± 4.7
Basal FSH (mIU/mL)	7.4 ± 3.1
AMH (ng/mL)	3.1 ± 2.6
Age of male partner at ICSI (y) ^a	39.8 ± 5.8
Ovarian stimulation protocol	
Clomiphene citrate (%)	15/137 (10.9)
Aromatase inhibitor (%)	13/137 (9.5)
GnRH antagonist (%)	55/137 (40.1)
GnRH agonist (%)	54/137 (39.4)

^aData are expressed as n or the mean ± standard deviation.

Spermatozoa were prepared from the partner's semen using two-layer Percoll density gradients. Normal-appearing spermatozoa were inseminated into oocytes, and then, zygotes and embryos were cultured in SAGE 1 Step Medium (Origio) under a humidified gas mixture of 5.5% CO₂, 5% O₂ and 89.5% N₂ at 37°C.

2.3 | Sperm preparation

Collected semen samples were centrifuged using two Percoll solutions (90% and 45%) for 20 minutes at 300 g and then centrifuged in washing buffer for 5 minutes at 300 g. Pelleted sperm was resuspended in Universal IVF Medium. Sperm concentrations and the percentage of motile sperm were counted by the Sperm Motility Analysis System (SMAS; DITECT) with 0.1 mL of Universal IVF Medium (Origio). Prepared semen with sperm concentrations <5 × 10⁶/mL or number of motile sperm <5 × 10⁶/mL were selected for c-ICSI or Piezo-ICSI.

2.4 | Conventional ICSI

The c-ICSI procedure was conducted using an Olympus inverted microscope (Olympus IX-73) equipped with a micromanipulator system (TAKANOME® MTK-1-03; NARISHIGE). Morphologically normal spermatozoa were immobilized in polyvinylpyrrolidone (7% PVP solution; Fujifilm Irvine Scientific) using an injection needle (K-MPIP-3130 Cook Medical), and oocytes were held by holding pipette (Kitazato Corporation) with the first polar body at twelve or 6 o'clock, and then, the injection needle was inserted at 3 o'clock and a single spermatozoon was injected after the first aspirating some ooplasm to break the oolemma.

2.5 | Piezo-ICSI

Piezo-ICSI was performed using ultrathin injection needles (PINU06-20FT; Prime Tech Ltd.) that had a flat-tip with 6 µm outer diameter and 5.1 µm inner diameter, as described by Hiraoka *et al*.⁵ Twelve microlitres of operating liquid was drawn into the center of the injection pipette. The

Piezo-assisted equipment used in this study was PIEZO PMM4G (Prime Tech Ltd.) equipped with a micromanipulator system (TAKANOME® MTK-1-03, NARISHIGE, Tokyo, Japan; Figure 1). The oocyte was restrained by the pipette, but the orientation of the first polar body was not considered when inserting the needle into the widely opened perivitelline space. Piezo pulses were generated with a speed setting of 2.0 and an intensity setting of 2.0 to penetrate the zona pellucida at three o'clock. An immobilized spermatozoon was aspirated then moved to the tip of the needle, which was inserted into the middle of the cytoplasm. The plasma membrane was punctured using a Piezo pulse (speed setting 2.0; intensity setting 1.0), and the spermatozoon was injected into the oocyte from the head. Cytoplasm was not aspirated into the injection needle.

2.6 | Evaluation of data

Fertilization rate (%) (2PN, 1PN, and 3PN), oocyte survival rate (%), and blastocyst formation per continuing pregnancy at day 5 or day 6 (%) were evaluated. Data were analyzed according to the age of women. Terms "aged" and "non-aged" were defined as women >35 or ≤35 years old, respectively, according to several reports which defined 35 years old as the border line of aged and non-aged women.⁶⁻⁸

2.7 | Morphological evaluation of cleaved embryos and blastocysts

Evaluation of blastocysts on D5 or D6 was performed according to the classification of Gardner and Schoolcraft⁹ and better than Grade 3BB embryos were designated good-quality blastocysts.

2.8 | Data analysis

Statistical analysis for numerical data was done using non-paired Student's *t* tests after the uniformity of dispersion was examined, and non-numerical data were evaluated using chi-squared tests. Statistical significance was set at *P* < .05.



FIGURE 1 Drive unit of a Piezo-micromanipulator PMM4G (Prime Tech Ltd., Ibaraki, Japan) attached to TAKANOME® (MTK-1-03, NARISHIGE, Tokyo)

3 | RESULTS

The mean age of all patients was 37.1 ± 4.7 years. The fertilization rate per oocyte with c-ICSI was 70.1% (331/472) and that with Piezo-ICSI was 75.4% (358/475) with no statistically significant difference. There was no significant difference in oocyte survival rate between the two treatments: c-ICSI, 94.3% (445/472) and Piezo-ICSI, 95.2% (452/475). The blastocyst development rates on D5/D6 were 50.2% (162/323) for c-ICSI and 54.9% for Piezo-ICSI (101/184), with similar results in both treatment groups (Table 2). The fertilization rates of Piezo-ICSI and c-ICSI in the ≤35-year-old age group were 78.0% (160/205) and 76.1% (156/205); the survival rates were 96.6% (198/205) and 97.1% (199/205); and the blastocyst development rates at D5/D6 were 61.7% (95/154) and 58.3% (84/144), respectively, with no statistically significant differences (Table 3). By contrast, for women aged >35 years, the fertilization rates of c-ICSI and Piezo-ICSI were 64.3% (171/266) and 74.8% (202/270) with a significant difference between treatments (*P* = .008). Oocyte survival rates of c-ICSI and Piezo-ICSI were 92.5% (246/266) and 94.1% (254/270), respectively, with no significant difference between the two groups. The blastocyst development rates on D5/D6 were significantly lower in the c-ICSI group (39.6%, 67/169) vs the Piezo-ICSI group (52.4%, 100/191) for women aged >35 years (*P* = .016; Table 4). As shown in Table 5, there were no significant differences in pregnancy rates (50.2%, 23/46 vs 45.2%, 14/31) and miscarriage rates (17.4%, 4/23 vs 21.4%, 4/14) between the c-ICSI compared with Piezo-ICSI groups.

4 | DISCUSSION

In this study, Piezo-assisted ICSI improved the fertilization and D5/D6 blastocyst development rates significantly in women aged >35 years old when it was compared with c-ICSI. However, there

TABLE 2 Clinical outcomes of conventional intracytoplasmic sperm injection (c-ICSI) and Piezo-ICSI for all patient ages

	c-ICSI	Piezo-ICSI	<i>P</i> value
No. of patients	137		
Age at ICSI (y) ^a	37.1 ± 4.7		
No. of mature oocytes	947		
Fertilization rate per oocyte (%)	387/472(82.0)	411/475(86.5)	NS
2PN (%)	331/472(70.1)	358/475(75.4)	NS
1PN (%)	23/472(4.9)	9/475(1.9)	<.01**
3PN (%)	6/472(1.3)	10/475(3.1)	NS
Oocyte survival rate (%)	445/472(94.3)	452/475(95.2)	NS
Blastocyst formation at day 5 or 6 (%)	162/323(50.2)	184/335(54.9)	NS

^aValues are expressed as the mean ± standard deviation.

***P* < .01.

TABLE 3 Clinical outcomes of conventional intracytoplasmic sperm injection (c-ICSI) and Piezo-assisted ICSI for patients ≤35 years old

	c-ICSI	Piezo-ICSI	P value
No. of women	45		
Age at ICSI (y)	31.0 ± 2.9		
No. of mature oocytes	410		
Fertilization rate per oocyte (%)	175/205 (85.4)	178/205 (86.8)	NS
2PN (%)	160/205 (78.0)	156/205 (76.1)	NS
1PN (%)	4/205 (2.0)	3/205 (1.5)	NS
3PN (%)	1/205 (0.5)	0/205 (0.0)	NS
Oocyte survival rate (%)	198/205 (96.6)	199/205 (97.1)	NS
Blastocyst formation at day 5 or 6 (%)	95/154 (61.7)	84/144 (58.3)	NS

were no statistically significant differences in any evaluated parameters when considering the whole patient group or the ≤35 years of age subgroup between Piezo-ICSI and c-ICSI, while a fertilization rate with 1PN in whole patients of Piezo-ICSI group was significant lower than c-ICSI group. This might be due to solid execution of penetrating oolemma by Piezo-ICSI. Piezo-ICSI is a technique that improves the fertilization rate of mouse, rabbit, equine, and bovine oocytes. It was reported to be more efficient than the c-ICSI method for fertilizing and obtaining more bovine embryos.¹⁰ In horses, Piezo-ICSI was associated with more rapid sperm component remodeling and meiotic resumption in the oocyte than c-ICSI and produced better quality blastocyst stage embryos.¹¹ The plasma membrane of

TABLE 4 Clinical outcomes of conventional intracytoplasmic sperm injection (c-ICSI) and Piezoassisted ICSI for patients >35 years old

	c-ICSI	Piezo-ICSI	P value
No. of women	92		
Age at ICSI (y)	40.0 ± 2.5		
No. of mature oocytes	536		
Fertilization rate per oocyte (%)	212/266 (79.7)	233/270 (86.3)	<.05**
2PN (%)	171/266 (64.3)	202/270 (74.8)	<.01*
1PN (%)	19/266 (7.1)	6/270 (2.2)	<.01*
3PN (%)	5/266 (1.9)	8/270 (3.0)	NS
Oocyte survival rate (%)	246/266 (92.5)	254/270 (94.1)	NS
Blastocyst formation at day 5 or 6 (%)	67/169 (39.6)	100/191 (52.4)	<.05**

*P < .01,

**P < .05.

TABLE 5 Clinical outcomes after embryo transfer and conventional intracytoplasmic sperm injection (c-ICSI) and Piezo-ICSI

	c-ICSI	Piezo-ICSI	P value
No. of blastocysts	46	31	
Age at ICSI (y)	35.0 ± 4.5	35.5 ± 5.4	NS
Pregnancy rate (%)	23/46 (50.0)	14/31 (45.2)	NS
Miscarriage rate (%)	4/23 (17.4)	4/14 (21.4)	NS

the mouse oocyte has high extendibility, and the ooplasm has low viscosity, and these factors lead to survival rates of <50% following c-ICSI.¹² In contrast, Piezo-ICSI can reduce damage to the oocyte's plasma membrane and achieve nearly 100% survival rates and high blastocyst development rates. For these reasons, Piezo-ICSI is considered highly effective for species exhibiting oocytes with delicate plasma membranes.^{13,14} Similar to our results, c-ICSI was reported to have a higher oocyte degeneration ratio than Piezo-ICSI for human oocytes.⁴ Hiraoka and Kitamura reported that Piezo-ICSI using an ultrathin needle could markedly reduce the size of the ruptured area in the oocyte's plasma membrane and improve oocyte survival and fertilization rates without detrimental effects on embryo development or implantation ability when compared with c-ICSI.⁵

It is widely accepted that both the quantity and quality of oocytes decline with a woman's age, leading to a lower success rate in ART.¹⁵ Several theories have been proposed to explain the decline in oocyte quality with age. The so-called "production line hypothesis" postulates that oocytes less susceptible to chromosomal non-disjunction are ovulated first, leaving poor quality oocytes to be ovulated later in life.¹⁶ However, this hypothesis does not explain why the order of oocyte differentiation induces the postulated differences in recombination frequency. Another theory invokes the age-related accumulation of damage from several mechanisms including oxidative stress, elevated FSH levels, and mitochondrial dysfunction.¹⁷⁻¹⁹

Our results indicate that changes in oocyte cell membrane viscosity may also be related to the woman's age. Finally, our findings suggest that Piezo-ICSI is particularly effective for patients aged >35 years undergoing ART, as Piezo-ICSI is less invasive to oocytes of aged women compared with c-ICSI.

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DISCLOSURES

Conflict of interest: Kohyu Furuhashi, Yoshimi Saeki, Noritoshi Enatsu, Toshiroh Iwasaki, Koichi Ito, Yuri Mizusawa, Yukiko Matsumoto, Shoji Kokeguchi, and Masahide Shiotani declare that they have no conflicts of interest.

Human rights statements and informed consent: This study was approved by the Institutional Review Board of Hanabusa Women's Clinic (IRB number Hanabusa Women's Clinic #7). We thoroughly explained that all obtained oocytes would be used only for this study and not for research on fertilization or for parthenogenetic activation. All procedures were conducted in accordance with the ethical standards of the response committee on human experimentation and with the Helsinki Declaration of 1964 and its larger amendments.

Animal studies: None of the authors performed any research on animal subjects in this study.

Written consent: Informed consent was obtained from all individual participants included in this study.

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