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Low Stretching Ability of Human Oolemma during Piezo-ICSI as a Risk Factor on Post-injection Survival and Implantation

Hiroshi Iwayama[1]*, Shinichi Hochi[2] and Masanori Yamashita[1]

[1]Yamashita Ladies' Clinic, Hyogo 651-0086, Japan
[2]Faculty of Textile Science and Technology, Shinshu University, Nagano 386-8567, Japan

Abstract: *The fate of human oocytes with impaired stretchability of the oolemma during piezo-ICSI was investigated in vitro (development into blastocysts in 5-day culture) and in vivo (implantation 4 weeks after embryo transfer). Oolemma of metaphase-II human oocytes was penetrated either before application of a piezo-pulse at stretching of less than 75% of the diameter (category-Low), or by a piezo-pulse when the oolemma was stretched over 75% of the diameter (category-High). Following in vitro culture, oocyte survival and developmental capacity to the blastocyst stage were compared between the two categories of oolemma stretchability. Moreover, we investigated whether oolemma stretchability was independently related as a risk factor of implantation using multivariate logistic regression. The post-injection survival rate of category-Low oocytes (73.6%) was significantly (P < 0.01) lower than that of category-High oocytes (99.4%). The blastocyst yield of category-Low oocytes (36.7%; calculated from surviving oocytes) was similar to that of category-High oocytes (44.5%). The low oolemma stretchability was found to be an independent risk factor of implantation (odds ratio 4.18, 95% confidence interval 1.04–16.74, P = 0.043). In conclusion, stretchability of human oolemma during piezo-ICSI affects post-injection survival, but not the developmental potential to blastocysts. Since oolemma stretchability also affects the implantation, we propose this parameter as a criterion for embryo selection in single embryo transfer.*
Key words: *Human oocytes, Implantation, Oolemma stretchability, Piezo-ICSI, Survivability*

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*To whom correspondence should be addressed.
e-mail: hiwayama@hotmail.co.jp

Introduction

Degeneration of human oocytes after intracytoplasmic sperm injection (ICSI) is an unavoidable phenomenon that clinical technicians experience. Several investigations have proposed a relationship between the post-ICSI oocyte degeneration and potential factors, including the location of the first polar body [1, 2], the volume of aspirated ooplasm [2], the skill of technicians [2, 3], the characteristics of patients [3, 4], the manner of ovarian stimulation [4], the size and sharpness of the injection pipette [5], and the system for ICSI [6, 7]. Among the potential factors investigated to date, the pattern of oolemma breakage has been especially noted to be associated with the survivability of ICSI oocytes [1, 2, 5, 8, 9].

Micromanipulators equipped with piezo-driven injection pipettes have been developed, originally for ICSI in mice [10], and have successfully been applied to ICSI in several mammalian species including humans [6, 7], rats [11] and cattle [12]. During piezo-ICSI, a blunt-ended injection pipette is mechanically advanced deep into the oocyte, extensively stretching the oolemma. Then, the oolemma is penetrated by the application of single piezo-pulse. Unexpectedly, a certain proportion of human oocytes is penetrated by the pipettes before application of piezo-pulse. One report showed that human oocytes penetrated with a blunt-ended pipette before full stretching of the oolemma were likely to undergo cytolysis, and that an equal proportion of good quality embryos was obtained from oocytes penetrated without oolemma stretching [9]. In that study, the effect of oolemma penetration on human ICSI oocytes was investigated only by 2-day culture after ICSI. Moreover, the impact of oolemma

stretchability during ICSI on implantation has not yet been clarified.

In the present study, the relationship between the ability of the human oolemma to stretch during piezo-ICSI and the subsequent developmental potential *in vitro* (development into blastocysts in 5-day culture) was investigated. In addition, the association of oolemma stretchability as the independent risk factor of implantation was analyzed using multivariate logistic regression.

Materials and Methods

Patients and preparation of gametes

This retrospective study involved 166 ICSI treatment cycles for 133 patients between September 2008 and December 2009. After informed consent, ICSI was administered for patients with either poor sperm parameters of husband's semen or very low fertilization rates in previous *in vitro* fertilization (IVF) cycles.

The mean age ± S.D. of patients at the retrieval cycles was 37.3 ± 4.2 years-old. Ovarian stimulation was performed using gonadotrophin-releasing hormone antagonist protocol in association with human menopausal gonadotrophin (hMG) and follicle stimulating hormone (FSH). Human chorionic gonadotrophin (hCG) was administered when the maximum diameter of two or more follicles reached 18 mm. Cumulus-oocyte complexes were retrieved by ultrasound-guided transvaginal follicle aspiration approximately 36 h after the hCG injection. Oocytes were denuded by pipetting in a human recombinant hyaluronidase solution (ICSI Cumulase®; MediCult, Jyllinge, Denmark), and incubated in potassium simplex optimized medium supplemented with amino acids [13] (Global®; IVFonline, Guelph, ON, Canada) and 0.5% (w/v) human serum albumin (HSA; Irvine Scientific, Santa Ana, CA, USA) at 37°C in a humidified air of 5% O_2, 5% CO_2 and 90% N_2 for 1 to 2 h prior to ICSI.

Freshly ejaculated semen was washed once with the Hepes-buffered human tubal fluid (Modified HTF-HEPES; Irvine Scientific) containing 0.5% HSA by centrifuging for 5 min at 300 × g. The semen pellet was carefully layered under 1.0 ml of Modified HTF-HEPES/HSA in a small round bottom tube and incubated for 30 to 60 min at 37°C to allow motile sperm to swim-up. The spermatozoa that swum up into the top 100-μl were collected. Immediately before ICSI, 3.5-μl sperm suspension was mixed with 3.5-μl Modified HTF-HEPES/HSA containing 7.5% polyvinylpyrrolidone (Irvine Scientific).

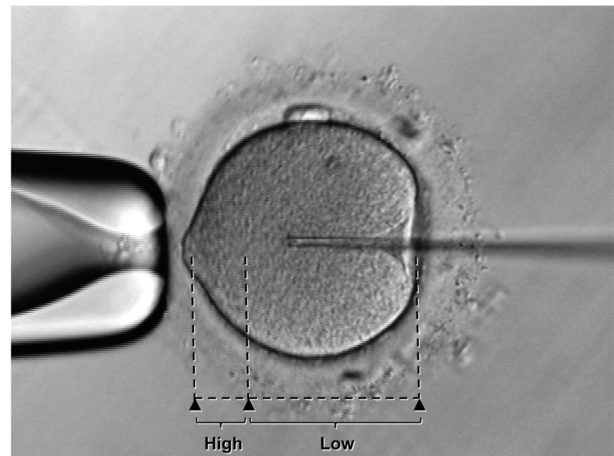

Fig. 1. Classification of human oocyte stretchability by a mechanically driven blunt-ended pipette. Oolemma were penetrated either before application of a piezo-pulse up to stretching of 75% (category-Low), or by a piezo-pulse when the oolemma was stretched beyond 75% (category-High). The diameter of individual oocytes was scaled as a 100% for determination of the penetration position.

Piezo-ICSI procedure

Piezo-ICSI was performed using a micromanipulator (Narishige Scientific Instruments, Tokyo, Japan) attached to a piezo impact driving unit (PMM-150FU; PrimeTech, Ibaraki, Japan), according to the method reported by Yanagida *et al.* [7] with a few modifications. Briefly, blunt-ended micropipettes for sperm injection with an outer diameter of 3 to 4 μm were prepared by breaking a heat-pulled glass capillary at the tip opening of the holding pipette. The spermatozoon was aspirated into the injection pipette tail-first with immobilizing by applying several piezo pulses (speed 3, intensity 3) to the tail just under the midpiece. The oocyte was held with the holding pipette with the first polar body (PB) located either at the 6 or 12 o'clock position. The zona pellucida was penetrated by several piezo pulses (speed 3, intensity 3), and the spermatozoon was repositioned at the tip of the injection pipette. The injection pipette was mechanically inserted deep into the oocyte with stretching the oolemma (category-High in Fig. 1), and a single piezo pulse (speed 1, intensity 1) was applied to penetrate the oolemma. The spermatozoon was gently injected into the ooplasm with a minimal amount of the surrounding medium. When the oolemma was penetrated before application of the piezo-pulse (category-Low in Fig. 1), the spermatozoon was carefully deposited into the ooplasm near the breakage position.

Embryo culture and transfer

The ICSI oocytes were cultured individually in 15-μl microdroplets of the Global®/0.5% HSA covered with mineral oil for a maximum 6 days at 37ºC in a humidified air of 5% O_2, 5% CO_2 and 90% N_2 (Day 0 was defined as the day of ICSI). After 16 to 18 h of ICSI, the number of oocytes surviving and exhibiting two pronuclei and two PBs (considered as normally fertilized) was recorded. Cleavage and development into blastocysts of the zygotes were recorded on Days 2 and 5, respectively. Blastocysts harvested on Day 5 were also subjected to transfer or cryopreservation on Day 5–6. Minimum volume cooling vitrification using Cryotop [14] was used as the cryopreservation procedure. Each blastocyst was morphologically scored according to Gardner’s criteria [15]. The excellent morphological quality of blastocysts was defined as: a developmental stage of “more than 3”, inner cell mass scores of “A” or “B” and trophectoderm scores of “A” or “B”.

The blastocysts, either freshly harvested on Day 5 or vitrified on Day 5–6, were transferred into the uteri of patients. To provide luteal support, the patients were administered with a vaginal suppository of progesterone (400 mg/day), intramuscular injection of progesterone (Progeston depot 125 mg/week; FujiPhalma, Tokyo, Japan), and transdermal estradiol (Estrana 1.44 mg/day; Hisamitsu Pharmaceutical, Tokyo, Japan). Implantations were confirmed by ultrasonic monitoring of the uterine cavity (gestational sac) 4 weeks after the embryo transfer (ET).

Statistical analysis

Variables associated with characteristics of patients (age, basal-FSH, dose of hMG/FSH and number of oocytes recovered) and ICSI technician were analyzed for incidence of category-Low oocytes. Moreover, for the relationship of oolemma stretchability to implantation, characteristics of transferred embryos (oolemma stretchability, ICSI technician, blastocyst quality and type of ET cycle) as well as the characteristics of patients were analyzed. First, a univariate logistic regression was used to calculate the crude odds ratio (OR) and 95% confidence intervals (CI), and both crude OR and CI were used in the inclusion of prospective variables in the multivariate analysis [16]. Inclusion of positive variables in the fitted model was achieved by forward and backward stepwise logistic regression selection or elimination of variables. The level of significance defined as the threshold for inclusion was the range of $0.05 \le P \le 0.1$, to avoid exclusion of potentially important variables [17]. Finally, the adjusted OR and 95% CI by multivariate logistic regression were presented as estimates of the relative risk. A value of $P < 0.05$ was considered to be significant. The predictive ability of the final logistic model (goodness-of-fit of model and the significance of model) was assessed by deviance residual, Pearson residual and likelihood ratio test. The values of significance considered were $P > 0.05$, $P > 0.05$ and $P < 0.05$, respectively.

Data of *in vitro* developmental capacity and efficiency from each category of oolemma stretchability were analyzed by the chi-square test. A value of $P < 0.05$ was considered statistically significant.

Results

Among 854 ICSI oocytes, 19.1% (n = 163; defined as category-Low) were penetrated by injection pipettes before application of the piezo pulse. In analysis by univariate logistic regression, only dose of hMG/FSH was positively associated with the incidence of category-Low oocytes; the other valuables were negatively associated. Consequently, a multivariate logistic regression model was not constructed, and relative risks could not be calculated for these variables (Table 1).

In vitro development of human ICSI oocytes with different oolemma stretchability is shown in Fig. 2. The post-injection survival rate of category-Low oocytes (73.6%, n = 163) significantly lower than that of category-High oocytes (99.4%, n = 691). Proportions of oocytes fertilized (84.1 and 82.5%), cleaved on Day 2 (82.7 and 76.7%) and developed into blastocysts on Day 5 (44.5 and 36.7%) were not significantly different between the two categories of oolemma stretchability (category-High and -Low oocytes, respectively), when the proportions were calculated from surviving oocytes. The final developmental efficiency *in vitro*, defined as the proportion of blastocysts on Day 5 (calculated from total oocytes injected), was lower in category-Low oocytes (27.0%) than in category-High oocytes (44.3%), due to the difference in the post-injection survival.

Transfer of 191 fresh or cryopreserved Day 5–6 blastocysts resulted in 59 implantations (30.9%) 4 weeks after ET. The implantation rates of blastocysts derived from category-High and -Low oocytes were 33.3 and 13.0%, respectively (Fig. 3). The independent risk factors associated with the implantation were investigated using multivariate logistic regression, as shown in Table 2. Univariate logistic regression revealed a significant association of implantation with

Table 1. Analysis of variables potentially associated with human oocytes with low oolemma stretchability

		Univariate logistic regression*[1]		
Variables	(range)	*P* value	Crude OR*[2]	95% CI*[3]
Patients' characteristics				
Age	(26–45 years old)	0.210	1.03	0.99–1.07
Basal-FSH	(1.0–15.2 mIU/ml)	0.304	0.96	0.88–1.04
Dose of hMG/FSH	(10.5–39.0 × 10^2 IU)	0.048	1.03	1.00–1.06
Oocytes recovered	(1–24)	0.912	1.00	0.97–1.04
Previous live birth: No vs. Yes (reference)		0.247	1.29	0.84–1.97
ICSI technician				
B vs. A (reference)		0.391	0.83	0.55–1.27
C vs. A (reference)		0.477	1.16	0.77–1.74

*[1]Multiple prospective valuables for multivariate analysis could not be selected. *[2] OR: odds ratio. *[3] 95% CI: 95% confidence interval of odds ratio.

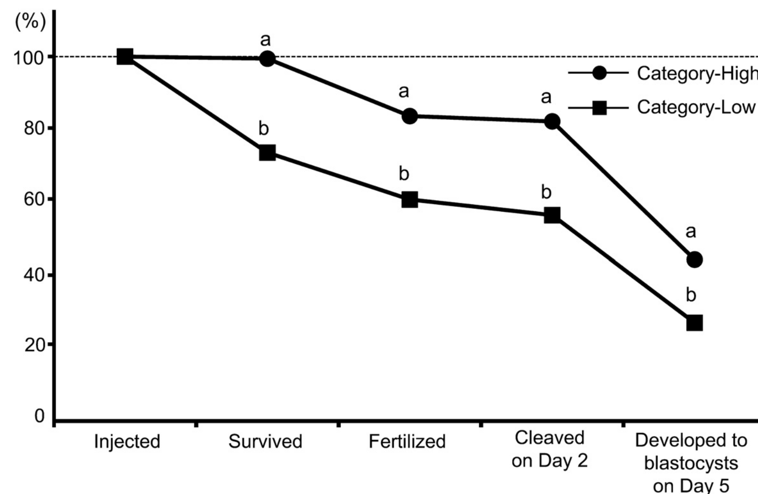

Fig. 2. Differential *in vitro* developmental efficiency of human oocytes with high/low stretchability during piezo-ICSI. Lower developmental efficiency through the term of the *in vitro* development was recorded in category-Low oocytes. a-b: Different letters within the same checkpoints indicate a significant difference, $P < 0.05$.

age, dose of hMG/FSH, oolemma stretchability, blastocyst quality, and type of ET cycle. In multivariate logistic regression with these five potential variables, three variables (oolemma stretchability, blastocyst quality, and type of ET cycle) were determined to be independent risk factors associated with implantation. This model showed a significantly good fit: deviance residual, $P = 0.547$; Pearson residual, $P = 0.409$; and likelihood ratio test, $P < 0.001$.

Discussion

In conventional ICSI with sharply beveled injection pipettes, the proportion of human oocytes penetrated before aspiration of ooplasm varies across the range of 9.4–20.4% [1, 2, 4]. Ruptures of oolemma probably occur when the penetration of zona pellucida is difficult [18–20]. Since blunt-ended pipettes actuated by a piezo-impact driving unit made it more feasible to penetrate the zona pellucida, accidental rupture of the oolemma which is unavoidable during the conventional ICSI does not occur during piezo-ICSI. The proportion

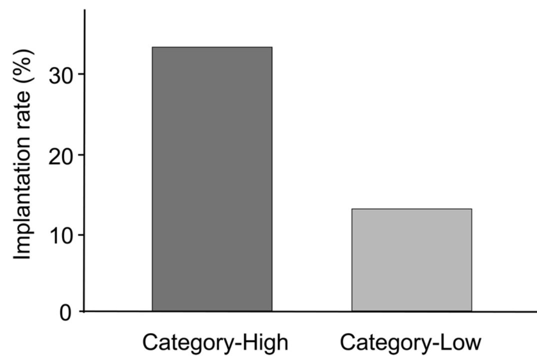

Fig. 3. Implantation rate of human blastocysts 4 weeks after transfer: Effect of oocyte stretchability during piezo-ICSI.

of category-Low oocytes in the present study was 19.1% (163/854). This incidence was not related to any variable because predictors could not be included together in a multivariable model to evaluate independent effects (Table 1). This suggests that the laboratory and clinical markers employed here are not good indicators for predicting the occurrence of human oocytes with the low stretchability. Oolemma stretchability may be determined by the characteristics of lipid bilayer; therefore, the lipid contents (phospholipids or fatty acids) of the follicular fluid in which each oocyte grew up requires further analysis. Alternatively, variations in oolemma stretchability may be attributable to incomplete spatial rearrangement of microfilaments in cortical and subcortical of metaphase-II oocytes [21], since the density and organization of microfilaments such as actin fiber and of microtubules are likely to occur during oocyte maturation [22].

The present study demonstrated that category-Low oocytes during the piezo-ICSI were sensitive to mechanical piercing with blunt-ended injection pipettes, which resulted in the lower survival rates (Fig. 2). This tendency of the lower survival rates in tandem with the decrease of oolemma stretchability is in agreement with previous reports of conventional ICSI [2, 5, 6] and piezo-ICSI [9]. It has been postulated that sealing (repair) of an opening in the oolemma may be facilitated by direct contact of the outer surfaces of the oolemma, which would take place during the post-ICSI recovery process in a fully-stretched oolemma [9]. Alternatively, a mechanical opening at the surface of the oolemma may be repaired by Ca^{2+}-dependent vesicle-vesicle fusion events, as reported in mouse 3T3 fibroblast cells [23] and sea urchin oocytes [24]. Consequently, a delay in resealing the penetrated oolemma may trigger an influx of a large amount of the surrounding medium (high sodium and low potassium) into the ooplasm [10]. Since the recovery from the disturbance of intracellular homeostasis is energy-dependent, the extent of the temporal intracellular changes may influence the viability of the oocytes. In murine ICSI procedure at low temperature (17–18°C), increased viscosity of the ooplasm and effectively blocked the pipette-made channel in the stretched oolemma, preventing an influx

Table 2. Determination of low oolemma stretchability during piezo-ICSI as a risk factor associated with implantation of transferred blastocysts

Variables	(range)	Univariate logistic regression			Multivariate logistic regression*[1]		
		P value	Crude OR*[2]	95% CI*[3]	*P* value	Adjusted OR*[2]	95% CI*[3]
Patients' characteristics							
Age	(26–45 years old)	0.015	1.13	1.05–1.12	0.211	1.06	0.97–1.16
Basal-FSH	(1.0–15.2 mIU/ml)	0.150	1.13	0.60–1.33			
Dose of hMG/FSH	(10.5–39.0 × 10^2 IU)	0.001	1.09	1.03–1.14	0.195	1.04	0.98–1.11
Oocytes recovered	(2–24)	0.107	0.95	0.89–1.01			
Previous live birth: No vs. Yes (reference)		0.722	1.11	0.55–2.26			
Transferred blastocysts' characteristics							
Oolemma stretchability							
Category-Low vs. -High (reference)		0.060	3.33	0.95–11.70	0.043	4.18	1.04–16.74
ICSI technician							
B vs. A (reference)		0.765	0.90	0.44–1.84			
C vs. A (reference)		0.570	1.25	0.58–2.73			
Blastocyst quality							
Fair vs. Excellent (reference)		< 0.001	7.67	3.78–15.54	0.001	4.54	2.08–9.93
Type of ET cycle							
Fresh vs. Cryopreserved (reference)		< 0.001	5.11	2.55–10.23	< 0.001	4.78	2.21–10.34

*[1] Goodness-of-fit of model: Deviance residual ($P = 0.547$), Pearson residual ($P = 0.409$), Significance of model: Likelihood ratio test ($P < 0.001$). *[2] OR: odds ratio. *[3] 95% CI: 95% confidence interval of odds ratio.

of the medium into the oocyte [10]. However, because room temperature could cause irreversible disruption of the metaphase II spindle in a human oocyte [25, 26], transient cooling to handling temperature should not be clinically used even if the viability after ICSI were to increase.

The proportions of ICSI oocytes fertilized and cleaved on Day 2 were not different between the two categories of oolemma stretchability. This finding agrees with a previous report by Yanagida *et al*. [9]. Moreover, the yield of blastocysts from category-Low oocytes was similar to that from category-High oocytes. Dumoulin *et al*. [2] reported that in conventional human ICSI, the development to the blastocyst stage was not affected by the type of oolemma breakage (20 and 19%, respectively), when oocytes were penetrated before and after ooplasmic aspiration. In the present study, due to the lower survivability of oocytes with category-Low stretchability, the developmental efficiency of blastocyst production from category-Low oocytes was significantly lower than that from category-High oocytes (Fig. 2). In other words, improving oocyte survival can lead to an increase in the developmental efficiency of those oocytes with lower stretchability of the oolemma.

To the best of our knowledge, this is the first report describing the implantation of human ICSI oocytes with low stretchability (Fig. 3). Oolemma stretchability was determined to be an independent risk factor of implantation by the present multivariate analysis (Table 2). If the transferred blastocysts originated from category-Low oocytes, the risk of failure in embryo implantation would increase 4.2-fold in comparison with category-High originated oocytes. The process of implantation for the human blastocysts consists of apposition, adhesion and invasion to the uterine epithelium, and is likely to be regulated by numerous factors: cell adhesion molecules, extracellular matrix (ECM) proteins, growth factors, cytokines, hormones, inflammatory factors and extracellular degrading matrix proteinases [27]. For instance, if expression of the ECM receptor integrin α_3, α_v, β_1, β_4 and β_5 on the trophectoderm membrane [28] or actin microfilaments combining with them [29, 30] was abnormal, an embryo may fail to implant. A characteristic of the human oolemma with low stretchability may be a congenital defect/phenotype presenting with aberrant factors in relation to implantation at the embryo membrane.

In conclusion, the stretchability of the human oolemma during piezo-ICSI affects the post-injection survival, but not the developmental potential to blastocysts. Since oolemma stretchability was evaluated as one of the potential risk factors of implantation, we propose this parameter as a criterion for embryo selection in single embryo transfer.

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