Flexibility of Oolemma is an Important Factor for Oocyte Survival after ICSI

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Abstract: *We examined the relationship between the breakage modes occurring during intracytoplasmic sperm injection (ICSI) and survival, fertilization and embryonic development rates after ICSI, and the morphologic quality of the oocytes before ICSI. The tip of each injection pipette was flattened to exclude the factor of tip shape. Oolemma breakage modes were grouped into four types according to oolemma flexibility. Approximately 8% of the mature oocytes treated with ICSI featured an inflexible oolemma, and 50% of such oocytes resulted in cytolysis, but no significant differences were observed in fertilization rates among the surviving oocytes, and no relationship could be traced between breakage modes and morphologic oocyte quality. The selection of oocytes with flexible oolemma is believed to be crucial in obtaining satisfactory results with ICSI, but it is not yet possible to distinguish oocytes that have inflexible oolemma from other oocytes before ICSI.*

Key words: *Oolemma breakage, ICSI, Survival rate, Fertilization rate, Piezo micromanipulator*

Intracytoplasmic sperm injection (ICSI) is an effective therapy for intractable infertility due to male factors and fertilization disorders after conventional in vitro fertilization (IVF) [1, 2]. ICSI differs significantly from other types of assisted reproductive technology (ART) in being an invasive procedure. Since ICSI begins with the insertion of a needle, damage to the oocyte cannot be avoided. Advances in ICSI technology have improved oocyte survival rates, boosting earlier survival rates of 50%-70% to current levels around 85%–90% [3–7], but ICSI technicians acknowledge the high probability of oocyte damage sometimes, even if the ICSI is performed properly and carefully. The oolemma of such oocytes is easily penetrated as soon as a needle is inserted into the oolemma. In earlier studies on oolemma breakage modes, technicians used injection needles with an acutely shaped tip [8, 9]. The processing of needle tips requires skill, and it is difficult to obtain tips of consistent sharpness. Since the modes in which oolemma break upon insertion of a needle are affected by the shape of the needle, we could apply these completely flattened tips with a piezo micromanipulator [10, 11] to this clinical trial to avoid such effects of sharper needles. We studied the different modes of oolemma breakage with regard to their relation to the microscopic morphologic characters of oocytes just before ICSI and their effects on fertilization and embryonic development following ICSI.

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Materials and Methods

This study involved 329 treatment cycles for which ICSI was performed at the Department of Obstetrics and Gynecology of Fukushima Medical University, between September 1997 and December 1998. ICSI was performed for patients with severe oligozoospermia and asthenozoospermia and for fertilization disorder in conventional IVF. The cases of azoospermia were excluded. A total of 1,740 oocytes were collected, of which 1,462 oocytes (84.0%) were mature (metaphase II oocytes). ICSI was performed on all mature oocytes. Survival and fertilization rates and the percentage of good quality embryos after ICSI were compared according to the breakage modes of oolemma. And the correlation of the breakage modes and morphologic assessments of oocytes before ICSI were discussed.

Preparation of oocytes and spermatozoa

Ovarian stimulation and oocyte preparation were performed as described by Yanagida *et al.* [5]. ICSI was

performed only for metaphase II oocytes. The cytoplasmic vacuoles were thought to be degenerative aspects and indicated a poor prognosis for fertilization. So the oocytes with the cytoplasmic vacuoles and ooplasma deformities were judged to be defective oocytes. Before ICSI, oocyte morphology was observed with an inverted microscope and classified as good (normal forms without defective findings) or defective (defective forms having vacuoles or deformities in the ooplasma. Survival rates, fertilization rates, and the percentage of good quality embryos after ICSI were then compared.

Preparation of spermatozoa was performed as described previously [5].

Method of ICSI

Instruments, oocyte holding pipette, injection pipette, and chamber were prepared according to the ICSI method described previously [5]. ICSI was performed with a piezo-micromanipulator (model PMM-MB-A, PRIME TECH Ltd, Tsuchiura city, Japan). Injection pipettes were formed with a micropipette puller (Model P-97IVF, Sutter Instrument Co., USA) and shaped to an external diameter of 5 micrometers. The tip of each pipette was completely flattened on a micro forge (MF79, Narishige, Tokyo, Japan). A spermatozoon with good motility and normal shape was immobilized by the application of several piezo pulses to its tail in a drop of PBS containing 8% polyvinylpyrrolidone (PVP-360, Sigma chemical, St. Louis, MO, USA) with the injection pipette just before ICSI. The immobilized sperm was drawn into the tip of the injection pipette, after which the pipette was allowed to penetrate the zona pellucida as piezo pulses were applied. The pipette was inserted into the oocyte by hand (without piezo pulses). The spermatozoon was injected into the oocyte after confirming breakage of the oolemma by the method described below, and the pipette was gently removed. This procedure was performed on the stage of a microscope warmed to 37°C.

Classification of oolemma breakage modes

The oolemma was broken by allowing the injection pipette to penetrate the zona pellucida and the oocyte. Breakage modes were classified into the following four types: type A, in which the oolemma was broken with little membranous stretching immediately on insertion of the pipette into the oocyte; type B, in which the oolemma was broken when the pipette was inserted into the oocyte to one-third of the diameter; type C, in which the oolemma was broken when the pipette was inserted as far as the center; and type D, in which the oolemma was not broken when the pipette was inserted to three-fourths of the diameter, but was broken when additional piezo pulses were applied (Fig. 1). The breakage types were determined by individuals performing and monitoring the ICSI on the display.

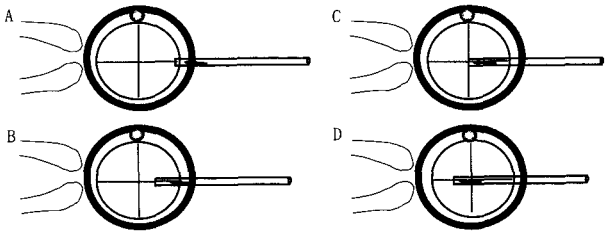

Fig. 1. Oolemma breakage mode. A: Rupture of oocyte membrane occurring just after insertion of the needle. B: Rupture of oocyte membrane occurring when the needle is inserted to 1/3 of the oocyte diameter. C: Rupture of oocyte membrane occurring when the needle is inserted to 1/2 of the oocyte diameter. D: Rupture of oocyte membrane occurring when the needle is inserted to 3/4 of the oocyte diameter after administration of additional piezo pulses.

Assessment of survival, fertilization and good quality embryo rates

Survival and fertilization rates were assessed 16–18 hours later, and the embryos were classified 40–44 hours later according to Veeck's classification by uniformity of blastomere and development of fragmentation into G1–G5 [12]. The number of good quality embryos, that is grades G1 and G2, was then recorded. Survival rates, fertilization rates and the percentage of good quality embryos for each breakage mode of the oolemma were compared. Poor quality embryos included G3, G4 and G5 embryos.

The results were statistically evaluated by the χ^2 test, with $p<0.05$ considered to indicate significance.

Results

Of the 1,462 oocytes treated with ICSI, 110 (8%) exhibited type A, 202 (14%) exhibited type B, 89 (6%) exhibited type C, and 1061 (72%) exhibited type D breakage modes (Table 1). Type A oocytes (47%) demonstrated significantly lower survival rates than other oocytes ($p<0.001$), whereas type D oocytes (94%) demonstrated significantly higher survival rates than types B and C ($p<0.001$). There were significant differences between type A and other types in the fertilized oocytes ($p<0.001$) and the good quality embryos ($p<0.01$).

Table 1. Survival, fertilization and embryo quality after ICSI in the breakage mode of oolemma

Mode of breaking	No. (%) of oocytes injected	No. (%) of surviving oocytes	No. (%)[1)] of fertilized oocytes	No. (%)[2)] of good quality embryos
A	110 (8)	52 (47)[a]	37 (34)[c]	19 (17)[d]
B	202 (14)	157 (78)	129 (64)	64 (32)
C	89 (6)	68 (76)	61 (69)	26 (29)
D	1061 (72)	1001 (94)[b]	761 (72)	361 (34)
Total	1462	1278 (87)	974 (68)	470 (32)

1) Fertilized oocytes among injected oocytes.
2) Good quality embryos among injected oocytes.
a) There were significant differences between A and B, A and C, and A and D ($p<0.001$).
b) There were significant differences between B and the others ($p<0.001$).
c) There were significant differences between A and the others ($p<0.001$).
d) There were significant differences between A and the others ($p<0.01$).

Table 2. Results of ICSI according to quality of oocytes

Quality of oocytes	Mode of breaking	No. (%) of Oocytes injected	No. (%) of surviving oocytes	No. (%) of fertilized oocytes	No. (%) of good quality embryos
Good	A	88 (7)	41 (47)[a]	28 (32)[f]	11 (13)[h]
	B	170 (14)	133 (78)[b]	110 (65)	59 (35)
	C	72 (6)	57 (79)[c]	48 (67)	20 (28)
	D	914 (73)	868 (95)	656 (72)	316 (35)
	Total	1244	1099 (88)	842 (68)	406 (33)
Poor	A	22 (10)	11 (50)	9 (41)	5 (23)
	B	32 (15)	24 (75)	19 (59)	5 (17)
	C	17 (8)	12 (65)[d]	8 (47)	6 (35)
	D	147 (68)	133 (90)[e]	105 (71)[g]	45 (31)
	Total	218	179 (87)	141 (65)	61 (28)

Poor quality oocytes were the oocytes that were detected with a vacuole or deformity in the metaphase II oocytes. The metaphase II oocytes except A were all classed as of good quality.
a) There were significant differences between A and B, A and C, and A and D ($p<0.001$).
b) There were significant differences between B and D ($p<0.001$).
c) There were significant differences between B and C, and C and D ($p<0.05$).
d) There were significant differences between A and C ($p<0.05$).
e) There were significant differences between A and D, and B and D ($p<0.001$).
f) There were significant differences between A and others ($p<0.001$).
g) There were significant differences between A and D ($p<0.01$).
h) There were significant differences between A and B ($p<0.001$), and A and C ($p<0.05$).
No significant differences were observed among all other pairs in the table.

Oocyte morphology before ICSI was good for 1,244 oocytes (85%) and defective for 218 oocytes (15%) (Table 2). Both forms most commonly exhibited type D breakage mode, and the ratios of the type A-D oocytes were identical to those obtained overall. For both good and defective forms, survival rates were significantly lower for type A oocytes and significantly higher for type D oocytes (no significant differences were found between A and B or C and D in the defective group). For the good quality oocyte group, the fertilization rate and good quality embryo rate were significantly lower in type A than in other types. For the poor quality oocyte group, the fertilization rate of type D (71%) was significantly higher than others. No significant differences between good and defective oocytes or among oolemma breakage modes were observed in the survival rate, fertilization rate and the percentage of good quality embryos.

Figure 2 showed the fertilization rate of injected oocytes and the percentage of good quality embryos obtained from fertilized oocytes. No significant differ-

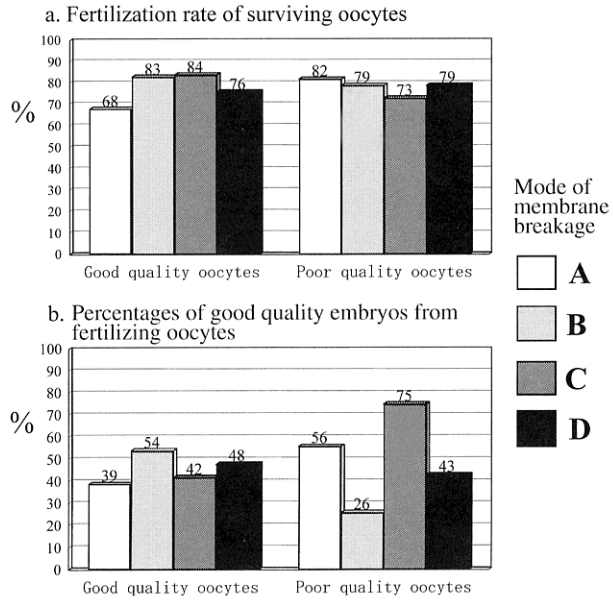

Fig. 2. The fertilization rate and the percentage of good quality embryos after ICSI in good/poor quality oocytes (recalculated values). a. Fertilization rate of surviving oocytes. b. Percentage of good quality embryos from fertilized oocytes. No significant differences were observed among the pairs in the graphs.

ences were observed between good and defective oocytes or among oolemma breakage modes in the fertilization rate and the percentage of good quality embryos.

Discussion

During many ICSI repetitions, we noticed similarities in the modes of penetration of the zona pellucida, while modes of oolemma breakage appeared quite diverse. Central to this study was the use of a piezo-micromanipulator for ICSI, first used by Kimura *et al.* [10] in mice. Application of this procedure to human ICSI has already been reported [5, 11]. ICSI performed with a piezo-micromanipulator involves injection pipettes with completely flat tips, and offers features such as freedom from deformation of oocytes during ICSI, the possibility of penetrating the zona pellucida and breaking the oolemma separately, and no need for the suction of the ooplasma by negative pressure to break the oolemma. With this method it was thought that the elasticity of the oocyte membrane was able to be evaluated better than with the conventional ICSI method with a sharpened needle. Different oolemma breakage modes have already been reported [8, 9], but those studies involved the use of needles with an acute tip, creating the possibility that the results are influenced by the sharpness of the tip.

Of 1,462 oocytes treated with ICSI, 72% featured oolemma with good flexibility (type D) and 8% had an oolemma with very poor flexibility (type A); that is, the oolemma was broken as soon as the pipette was inserted. Oocytes with inflexible oolemma had significantly lower survival rates, whereas those with flexible oolemma had significantly higher survival rates. These results corroborate those reported by Nagy *et al.* [8] and Palermo *et al.* [9].

The fertilization rate and the percentage of good quality embryos as shown in Table 2 were low in the oocytes with inflexible oolemma, the reason being that the number of surviving oocytes was small. Nevertheless, no relationship was observed between oolemma flexibility and the fertilization rates of the surviving oocytes or the percentage of good quality embryos obtained from the fertilized oocytes, and no differences were observed among the modes of oolemma breakage. These results are identical to those reported by Nagy *et al.* [8]. The oocytes with inflexible oolemma easily became cytolytic, but the surviving oocytes after ICSI had the same fertilization and embryo development abilities as the oocytes with flexible oolemma. It seemed that only membrane function was disordered in the oocytes with inflexible oolemma.

Among the factors potentially influencing oolemma flexibility, we focused on the morphologic quality of the oocytes before ICSI to detect the oocytes with a poor prognosis [13]. Excessive granularity, cytoplasmic vacuoles and a refractile body in the abnormal morphology of the oocytes were sometimes found in ooplasma under the microscope. These findings were thought to be degenerative aspects [13]. Especially the cytoplasmic vacuoles indicated a poor prognosis for fertilization [13]. So the oocytes having cytoplasmic vacuoles and deformity were defined as defective oocytes in this study. The ratios of breakage modes did not vary with morphologic differences in the oocytes before ICSI. These results indicate that the morphologic quality of the oocytes does not affect oolemma flexibility. The fertilization rate and the percentage of good quality embryos from defective oocytes with an inflexible oolemma were the same as those from good oocytes. Alikani *et al.* [14] also reported that fertilization rates and early embryonic development were uninfluenced by the morphologic quality of oocytes. We examined the possibility that morphologic anomalies could indicate oolemma flexibility, but no relationship was observed between oolemma

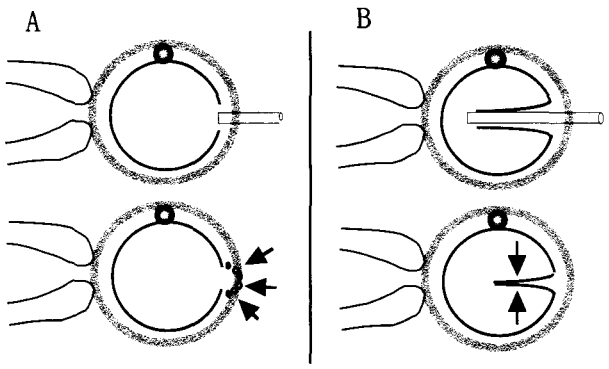

Fig. 3. The mechanisms of oolemma resealing. The arrow indicates the portion that is resealed. A) Mechanism for Type A: Vesicles released from the ooplasma surrounding the opening fuse with each other and reseal the hole. B) Mechanisms for Types D: The injection pipette penetrates the oolemma after the oolemma is pushed into the ooplasma. The extended regions of the membrane bond and fuse with each other after the pipette is extracted. The mechanisms of types B and C are mixed A and B.

flexibility and the morphologic quality of oocytes.

Two processes may be involved in resealing the hole formed in the oolemma by the insertion of the injection pipette in ICSI (Fig. 3). For type B, C, or D oocytes, in which the pipette penetrates after the oolemma is pushed into the ooplasma some distance, the extended oolemma may bond and fuse with each other after the needle is extracted [10]. The resealing occurs quickly. For type A oocytes, in which the oocyte is penetrated as soon as a pipette is inserted, a larger hole is made in the oolemma and will be resealed by the fusion of vesicles emitted from the ooplasma surrounding the hole, as reported by Miyake *et al.* [15] concerning endothelial cells. Such a phenomenon was observed in sea urchin oocytes [16]. Since the hole is too large, the resealing is delayed or terminated, so the oocyte begins to degenerate.

In conclusion, approximately 8% of mature oocytes treated with ICSI had an inflexible oolemma, and 50% of these resulted in cytolysis after ICSI. It is not yet possible to distinguish such oocytes from other oocytes before ICSI. But the surviving oocytes after ICSI could fertilize and develop the same as the oocytes with a flexible oolemma.

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