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ASSISTED REPRODUCTION TECHNOLOGIES

Developmental ability of embryos produced from oocytes with fragile oolemma by intracytoplasmic sperm injection

Yamato Mizobe[1,2] · Naoto Oya[1] · Reiko Iwakiri[1] · Naomi Yoshida[1] · Yumi Sato[1] · Nanase Onoue[1] · Kazuchika Miyoshi[2] · Makoto Tokunaga[1] · Yuji Ezono[1]

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Abstract
Purpose In intracytoplasmic sperm injection (ICSI) of oocytes with a fragile oolemma (fragile oocytes), breakage can occur at injection. In this study, we produced embryos from oocytes with a fragile and normal oolemma (normal oocytes) by ICSI and compared their ability to be fertilized and develop in vitro. We also investigated whether fragile oocyte-derived embryos could implant after blastocyst transfer to determine whether fragile oocytes should be used for assisted reproductive technology treatment.
Methods Oocytes were divided into three groups—normal oocytes from cycles containing no fragile oocytes (group A), normal oocytes from cycles containing at least one fragile oocyte (group B), and fragile oocytes (group C), and their fertilization abilities after ICSI and the developmental abilities of resultant embryos were compared.
Results The fertilization rate in group C (65.3 %) was significantly ($P < 0.01$) lower than those in groups A (84.6 %) and B (86.9 %), and the degeneration rate in group C (24.2 %) was significantly ($P < 0.01$) higher than those in groups A (0.71 %) and B (0.28 %). However, there were no significant differences in the blastocyst formation rates (59.7–67.5 %) of embryos among the different groups. In addition, the pregnancy rate after transfer of blastocysts in group C (50.0 %) was not significantly different from those in groups A (35.6 %) and B (45.8 %).
Conclusions The fertilization ability after ICSI of fragile oocytes is lower than that of normal oocytes but the resultant embryos have the same developmental ability as those of normal oocyte-derived embryos.

Keywords Embryo · Intracytoplasmic sperm injection · In vitro fertilization · Oocyte plasma membrane · Oolemma · Time-lapse incubator

Capsule In intracytoplasmic sperm injection (ICSI) of oocytes with fragile oolemma, breakage can occur at injection. We produced embryos by ICSI of oocytes with fragile and normal oolemma, compared fertilization and development in vitro, and implanted fragile oocyte-derived embryos after blastocyst transfer to determine usability of fragile oocytes in assisted reproduction.

✉ Yamato Mizobe
lca.aiiku.lab.3f@gmail.com

[1] Aiiku Ladies Clinic, 1-40-2 Komatsubara, Kagoshima 891-0114, Japan

[2] The United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

Introduction

Intracytoplasmic sperm injection (ICSI) is a method of in vitro fertilization (IVF) in which a single sperm is introduced directly into the cytoplasm of a matured oocyte by penetration of the plasma membrane known as the oolemma. The ICSI method is considered an essential technique in modern assisted reproductive technology (ART) [1–4]. However, degeneration of oocytes occurs after ICSI even when maximum care is exercised. Rosen et al. [5] suggested that oocyte degeneration is a common phenomenon experienced by all technicians who perform ICSI. Van Steirteghem et al. [6] reported that an average of 10 % of oocytes may be lost because of degeneration as a result of the procedure. Degeneration of oocytes is particularly evident when the oolemma is very fragile, resulting in sudden breakage of the membrane during ICSI [7, 8]. The observation that oocytes with a fragile oolemma (fragile oocytes) degenerate easily can be explained by the absence of the protective effect of the funnel responsible for sealing the breach. The discontinuity of the oolemma might

affect the cortical component of the cytosol and consequently the oocyte cytoskeleton, leading to disturbances in the microtubules responsible for chromatid segregation and expulsion (in the form of polar body) at the second meiotic division [7].

There are patients in which most of the retrieved oocytes have the fragile oolemma, resulting in a high degeneration rate after ICSI [7, 9]. In such cases, we sometimes have no choice but to transfer embryos derived from fragile oocytes. In general, they are cultured for 5 to 6 days and then, embryos that have reached the blastocyst stage are cryopreserved and later warmed to be transferred after the next cycle because the pregnancy rate after the transfer of cryopreserved blastocysts is higher than that of fresh early embryos [10, 11]. For these reasons, we need to examine whether blastocysts can be obtained from fragile oocytes and the blastocysts can be implanted after cryopreservation.

In this study, therefore, we produced embryos from oocytes with fragile or normal oolemma by ICSI and compared their ability to be fertilized and developed in vitro. In addition, we investigated whether fragile oocyte-derived embryos could be implanted after the blastocyst transfer process to determine whether fragile oocytes should be used for ART treatment.

Materials and methods

Patients

Between March 2014 and April 2015, we examined the outcome of 133 retrieval cycles. The mean age ± standard deviation (range) of patients at the retrieval cycles was 36.2 ± 4.1 years (27–47 years). Retrieved oocytes were fertilized by ICSI (76 retrieval cycles) or split cycle combined ICSI and conventional IVF (57 retrieval cycles) using standard techniques [2, 3]. For split cycle, oocytes were randomized to conventional IVF or ICSI.

Ethical considerations

All study participants provided informed consent, and the study design was approved by the appropriate ethics review boards. Written informed consent for their treatment and for their outcomes to be described was obtained from all patients.

Ovarian stimulation and oocyte retrieval

Ovarian stimulation was performed using the gonadotropin-releasing hormone (GnRH) analog buserelin acetate (Fuji Pharmaceutical Co., Ltd., Tokyo, Japan) and the short or long protocol together with follicle-stimulating hormone (FSH) and human menopausal gonadotropin (hMG) (ASUKA Pharmaceutical Co., Ltd., Tokyo, Japan) stimulation. Human chorionic gonadotropin (hCG; Fuji Pharmaceutical Co., Ltd.) or leuprolide were administered when the maximum diameter of two or more follicles reached 18 mm. Cumulus/oocyte complexes were retrieved by ultrasound-guided transvaginal follicle aspiration at approximately 36 h after hCG injection. The concentration of the anti-Müllerian hormone (AMH) was measured during a cycle before oocyte retrieval. Values for FSH and hMG represented the amount of these medications used during the oocyte retrieval cycle. The concentration of estradiol was measured 2 days before oocyte retrieval and used as an index for determining the retrieval date. The retrieved oocytes were pre-cultured for 3 h in a 10 % serum-added human tubal fluid (HTF) (NAKA Medical, Tokyo, Japan). After pre-culture, oocytes were denuded by pipetting in 80 U/ml hyaluronidase solution (NAKA Medical).

ICSI of oocytes

A sperm was immobilized in 7 % polyvinylpyrrolidone (PVP) solution (NAKA Medical), and then ICSI was performed in a drop of 20 % serum-added HEPES-HTF (NAKA Medical). We used an injector, IM-11-2 (Narishige, Tokyo, Japan), and injection pipettes, K-MPIP-3130 (Cook Medical, Bloomington, IN, USA).

Oocytes whose oolemma was broken at the moment when the injection pipette was inserted into the ooplasm without aspiration during ICSI were defined as fragile oocytes (Fig. 1a). Oocytes whose oolemma was broken by aspiration after the insertion of an injection pipette into the ooplasm were defined as normal oocytes (Fig. 1b). Of 133 cycles examined in this study, 83 cycles (62.4 %) had only normal oocytes and 50 cycles (37.6 %) had at least one fragile oocyte. Normal oocytes from the former and latter were defined as groups A and B, respectively. Fragile oocytes from the latter were defined as group C.

Culture of embryos

All embryos were transferred to 30 μl of One Step Medium (NAKA Medical) under sterile mineral oil and cultured under 5 % CO_2, 5 % O_2, and 90 % N_2 at 37 °C to the blastocyst stage using EmbryoScope™ (VitroLife, Göteborg, Sweden).

The rates of fertilization, degeneration, blastocyst formation, and good-quality blastocyst formation among the groups A, B, and C were compared. Fertilization was confirmed by the presence of two pronuclei and two polar bodies after ICSI. Oocytes in which ooplasm shrank and darkened within a few hours of ICSI were determined as degenerated oocytes. Good-quality blastocysts were defined as those scoring B or higher for both inner cell mass and trophectoderm grades [12]. In addition, we observed the developmental morphology of the early embryos and compared the second polar body extrusion time, pronuclei appearance, and disappearance times, and the

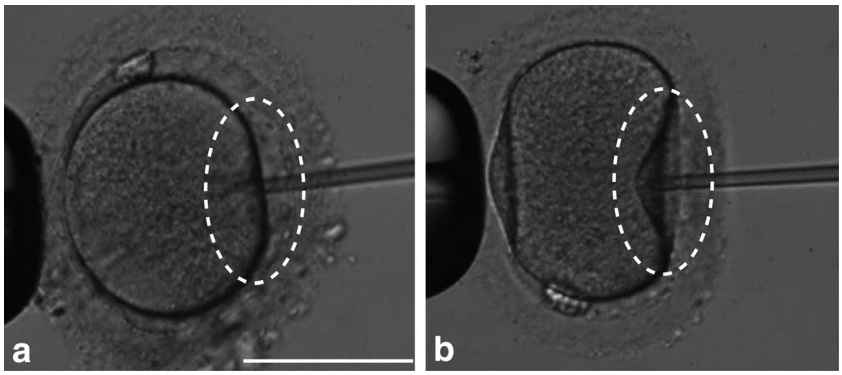

Fig. 1 Fragile (**a**) and normal (**b**) oocytes. As shown in the *circles*, oolemma was broken by the insertion of an injection pipette in fragile oocytes. *Scale bar* 100 μm

first and second division times among the three groups because these factors can be used to select good-quality embryos [13–15].

Transfer of blastocysts

Fragile oocyte-derived blastocysts were cryopreserved and then transferred to 13 patients for 16 cycles after the next hormone replacement therapy cycle. Their pregnancy rate was compared to that of 78 patients for 132 cycles in which normal oocyte-derived blastocysts were cryopreserved and then transferred at the same time as the first group. Cryopreservation of the embryos using a Cryotop® (Kitazato, Shizuoka, Japan) was performed using established methods [16, 17]. After thawing, a single blastocyst transfer was performed and clinical pregnancy rates were determined based on the presence of a gestational sac as visualized by ultrasound at 3 weeks after embryo transfer.

Statistical analysis

The statistical significance of the differences in the mean values between the two populations was assessed with Student's *t* test (Table 1). Statistical analysis was performed using the Bonferroni corrected chi-squared test with continuity correction (Tables 2 and 3) or Kruskal–Wallis analysis of variance (Table 4). $P < 0.05$ was considered statistically significant.

Results

Patients

As shown in Table 1, the number of oocytes retrieved (12.6 ± 5.5) and matured (11.0 ± 4.9) in the patients who provided at least one fragile oocyte (groups B and C) was significantly ($P < 0.05$) higher than that (9.8 ± 7.5 and 8.4 ± 6.7) in those who provided only normal oocytes (group A), respectively. The estradiol level in groups B and C (7549 ± 6310 pg/ml) was significantly ($P < 0.05$) higher than that (5406 ± 4525 pg/ml) in group A. There were no significant differences between the two patient groups in terms of the other factors.

Fertilization, degeneration, and blastocyst formation rates

As shown in Table 2, groups A and B showed significantly ($P < 0.01$) higher fertilization rates (84.6–86.9 %) than group C (65.3 %). The degeneration rates in groups A and B (0.28–0.71 %) were significantly ($P < 0.01$) lower than those in group C (24.2 %). However, there were no significant differences in the rates of blastocyst formation (59.7–67.5 %) and good-quality blastocyst formation (58.0–64.9 %) among the groups.

Developmental process of early embryos

The second polar body extrusion time in group C (2.87 ± 0.84 h) was significantly ($P < 0.05$) shorter than those in groups A and B (3.18 ± 0.95–3.32 ± 0.99 h) (Table 4). There were no significant differences among the groups in terms of the other factors.

Pregnancy rates

As shown in Table 3, there were no significant differences in the pregnancy (35.6–50.0 %) and miscarriage (15.4–25.9 %) rates among the groups.

Discussion

The results of this study showed that the numbers of oocytes retrieved and matured and the estradiol level on the 2 days before oocyte retrieval in patients with fragile oocytes are significantly higher than those in patients without fragile oocytes. In ART, we usually perform controlled ovarian stimulation to retrieve multiple oocytes. Joo et al. [18] reported that serum estradiol levels on the day of hCG administration

Table 1 Patient background

	Group A (83 cycles)	Groups B and C (50 cycles)
Age (years)	36.7 ± 4.0	35.3 ± 4.1
No. of oocytes retrieved	9.8 ± 7.5*	12.6 ± 5.5**
No. of matured oocytes	8.4 ± 6.7*	11.0 ± 4.9**
FSH (IU)	1198 ± 225	1188 ± 309
hMG (IU)[a]	1030 ± 724	843 ± 350
E2 (pg/ml)[b]	5406 ± 4525*	7549 ± 6310**
AMH (ng/ml)[c]	4.1 ± 3.5	4.8 ± 4.0

Data are presented as the mean ± standard deviation

*, **Values with different superscripts are significantly different ($P < 0.05$)

FSH follicle-stimulating hormone, *hMG* human menopausal gonadotropin, E_2 estradiol, *AMH* anti-Müllerian hormone

[a] Because hMG was not measured in 2 patients, data derived from 81 patients were shown in group A

[b] Because E_2 was not measured in 5 patients, data derived from 78 patients were shown in group A

[c] Because AMH was not measured in 16 and 7 patients, data derived from 67 and 43 patients were shown in group A and groups B and C, respectively

(2 days before oocyte retrieval) have a concentration-dependent effect on the number of oocytes retrieved, and the number of oocytes increases with increasing estradiol levels. This is consistent with our result. On the other hand, Otsuki et al. [19] reported that high serum estradiol levels on the day of hCG administration significantly increased the incidence of smooth endoplasmic reticulum clusters (sERCs) in oocytes and the presence of sERCs significantly decreased the pregnancy rates after the transfer of ICSI oocytes, suggesting that high estradiol levels in patients are associated with the abnormal morphology of human ooplasm. Therefore, the high estradiol level in patients might have increased the incidence of fragile oocytes in this study. Further investigation will be required to clarify this point.

In this study, the fertilization rate after ICSI of fragile oocytes was lower than that of normal oocytes because of the increased rate of degenerated oocytes. This result is consistent with previous reports in which oocyte degeneration was found to be closely related to the characteristics of the oolemma [7, 8]. However, once fragile oocytes were fertilized without degeneration, the developmental ability into the blastocysts of resultant embryos was compared favorably with that of normal oocyte-derived embryos. Therefore, the avoidance of oocyte degeneration after ICSI is the most important to produce blastocysts from fragile oocytes efficiently. A typical oolemma reaction associated with high degeneration rates is sudden breakage when pushing the injection pipette into the oocyte initially [7, 20]. In order to overcome abnormal break-

Table 2 Effects of oolemma properties on fertilization after ICSI and subsequent embryonic development

	Group A	Group B	Group C
No. of oocytes injected	422	351	95
No. (%)[a] of oocytes fertilized	357 (84.6)*	305 (86.9)*	62 (65.3)**
No. (%)[a] of oocytes degenerated	3 (0.71)*	1 (0.28)*	23 (24.2)**
No. of embryos cultured	348[d]	305	62
No. (%)[b] of blastocysts	231 (66.4)	206 (67.5)	37 (59.7)
No. (%)[c] of good-quality blastocysts	134 (58.0)	123 (59.7)	24 (64.9)

*, **Values with different superscripts are significantly different ($P < 0.01$)

ICSI intracytoplasmic sperm injection

[a] Percentage per oocytes injected

[b] Percentage per embryos cultured

[c] Percentage per blastocysts

[d] Nine fertilized oocytes were cryopreserved or transferred on day 1, 2, or 3

Table 3 Clinical pregnancy after the transfer of blastocysts derived from normal or fragile oocytes

Group	No. of embryos transferred	No. (%)[a] of pregnancies	No. (%)[b] of miscarriages
A	73	26 (35.6)	4 (15.4)
B	59	27 (45.8)	7 (25.9)
C	16	8 (50.0)	2 (25.0)

[a] Percentage per embryos transferred

[b] Percentage per pregnancies

age of the fragile oolemma, Palermo et al. [7] proposed a tangential penetration of the zona pellucida in which the injection pipette is pressed against the zona to penetrate it at 6 or 12 o'clock and then the oocyte is rotated to position the pipette tip at 3 o'clock and the pipette tip is pressed against the oolemma. Moreover, creating a microhole on the zona pellucida of the fragile oocyte by laser beam prior to ICSI provided a less traumatic penetration of the injection pipette into the ooplasm and resulted in lower degeneration and higher embryo development rates [21]. On the other hand, various efforts have also been made to avoid degeneration of normal oocytes after ICSI. To reduce oocyte vulnerability by preventing deformation during ICSI, a laser-assisted opening [20] or partial thinning [22] of the zona pellucida before penetration has been applied. A piezo-micromanipulator is useful for decreasing the degeneration rate of ICSI oocytes [23, 24]. Another report showed that it is possible to improve ICSI outcomes by using an ultra-thin micropipette [25]. These techniques would improve the fertilization rate after ICSI of fragile oocytes, which brings about the efficient production of blastocysts from them.

From the time-lapse observation, it was shown that the second polar body extrusion time after ICSI of fragile oocytes is significantly shorter than that of normal oocytes. In embryonic development, there is an appropriate time for extrusion of the polar body, formation and disappearance of the pronucleus, and cleavage [15, 26, 27]. The results of this study suggest that fragile oocytes cannot extrude the second polar body within an appropriate timeframe, which is probably associated with their abnormal oolemma. Palermo et al. [7, 28] also reported that fragile membranes have the reduced ability to extrude the second polar body. It is unclear whether this phenomenon is related to the increased degeneration rate of fragile oocytes. However, the phenomenon would not affect the subsequent fertilization and developmental process of fragile oocytes because the mean times for abutment of male and female pronuclei, syngamy, first cleavage, and second cleavage of fragile oocytes were not different from those in normal oocytes and each time was similar to that previously reported [15, 26, 27]. The results of this study indicated that the pregnancy and miscarriage rates after the transfer of fragile oocyte-derived blastocysts are at the same level as those of normal oocyte-derived blastocysts, although the number of fragile oocyte-derived blastocysts transferred was limited to 16. These results suggest that fragile oocyte-derived blastocysts have the same ability to be implanted and develop into babies as that of normal oocyte-derived blastocysts. In addition, there were no significant differences in the fertilization rates of oocytes, blastocyst formation rates of embryos, and pregnancy rates between groups A and B. Therefore, the presence of fragile oocytes would not influence the quality of normal oocytes in the same cycle.

In conclusion, this study demonstrated that fragile oocytes are eligible for ART because the fertilization ability after ICSI of fragile oocytes is lower than that of normal oocytes but resultant embryos have the same developmental ability as that of normal oocyte-derived embryos. In the future, the establishment of a method that increases the fertilization rate of fragile oocytes will be essential for their use in ART treatment efficiently.

Table 4 Effects of oolemma properties on the developmental process of early embryos

	Group A	Group B	Group C
No. of embryos observed	348	305	62
No. of embryos assessed	348	294[a]	57[a]
Second PB extruded ± SD (h)	3.18 ± 0.95*	3.32 ± 0.99*	2.87 ± 0.84**
Both PN abutted ± SD (h)	7.70 ± 1.42	7.94 ± 1.59	7.81 ± 2.05
Syngamy ± SD (h)	23.52 ± 3.22	23.81 ± 3.33	23.93 ± 4.35
First cleavage commenced ± SD (h)	27.12 ± 3.77	27.21 ± 3.88	27.60 ± 4.92
Second cleavage commenced ± SD (h)	39.88 ± 4.56	38.99 ± 4.85	39.11 ± 5.12

*, **Values with different superscripts are significantly different ($P < 0.05$)

PB polar body, *PN* pronucleus, *SD* standard deviation

[a] Eleven and 5 embryos in which at least one of the events was not clearly observed were excluded in groups B and C, respectively

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants This study was approved by the Institutional Review Board of AIIKUKAI Medical Corporation.

Informed consent Written informed consent for their treatment and for their outcomes to be described was obtained from all patients.

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