INTRODUCTION

Oocyte degeneration following intracytoplasmic sperm injection (ICSI) occurs at a rate of ~5%-15%\(^1\,\,^3\) due to risk factors involving oocyte quality, such as oocyte cytoplasmic viscosity,\(^4,\,^5\) oolemma stretchability or flexibility,\(^6,\,^7\) and oolemma fragility.\(^8\) One study showed that the injection funnel volume after conventional ICSI is greater in normal fertilized oocytes than in degenerated oocytes.\(^4\)
while another study reported that the post-piezo-ICSI survival rate is lower with low oolemma stretchability than with high oolemma stretchability.\textsuperscript{9} Regarding embryonic development, there was a study that reported no association between the degree of oolemma fragility and embryonic development or pregnancy rates.\textsuperscript{8} Conversely, one study did find that the implantation rate following transfer was lower for a low stretchability oocyte-derived blastocyst than for a high stretchability oocyte-derived blastocyst.\textsuperscript{5}

The authors’ previous study demonstrated that the oolemma of human metaphase II (MII) oocytes from individual follicles with a low follicular fluid (FF) volume following the gonadotrophin-releasing hormone (GnRH) antagonist protocol were more likely to have a low stretchability during ICSI, suggesting a potential association between the FF volume with oolemma stretchability.\textsuperscript{9} However, the relationship between MII oolemma stretchability following the GnRH agonist protocol and the FF volume remains unclear.

The GnRH agonists and antagonists have different effects on the intraovarian autocrine and paracrine systems, as well as on the pituitary gland, to prevent a premature surge of luteinizing hormone during controlled ovarian stimulation (COS).\textsuperscript{10,11} Thus, the GnRH agonist suppresses gonadotrophin secretion through both pituitary desensitization and GnRH receptor downregulation.\textsuperscript{12} Conversely, a GnRH antagonist will rapidly inhibit gonadotrophin secretion by a competitive blockade of GnRH receptors.\textsuperscript{13} Accordingly, the potentially different effects of COS on ovarian folliculogenesis could lead to different responses and endocrine environments for maturing oocytes.\textsuperscript{14} Several studies have reported a relationship between the type of COS protocol and oocyte quality. Cytoplasmic viscosity\textsuperscript{5} and the morphology\textsuperscript{15} of the oocytes are not related to the COS protocol, whereas the quality of the zona pellucida is dependent on the COS protocol.\textsuperscript{16} Therefore, elucidating the impact of a COS protocol on oolemma stretchability during ICSI is crucial.

The aim of this study was to investigate the relationship between the FF volume that was aspirated from individual follicles and oocyte retrieval, oocyte maturity, the cumulus-oocyte complex (COC) grade, oolemma stretchability, fertilization, and embryonic development following the administration of a short GnRH agonist protocol, according to the outcome measures in the authors’ previous GnRH antagonist study.\textsuperscript{9}

2 MATERIALS AND METHODS

2.1 Patients

Data from 74 ICSI cycles that had been performed between April, 2016 and May, 2017 were reviewed retrospectively. The mean (±standard deviation) age of the women was 37.6 ± 4.0 (range: 28-44) years.

2.2 Controlled ovarian stimulation and oocyte retrieval

The COS was performed, as described previously.\textsuperscript{17} The patients were treated with the GnRH analog, acetate (Fuji Pharma Company, Ltd., Tokyo, Japan), and human menopausal gonadotropin (ASKA Pharmaceutical Company, Ltd., Tokyo, Japan) in accordance with the short GnRH agonist protocol. When at least two follicles had reached 18-20 mm in diameter (as determined by transvaginal ultrasonography), 5000 IU of human chorionic gonadotropin (hCG; Fuji Pharma Company) were administered. Oocyte retrieval was performed 36 hours after the hCG injection.

Individual follicles were aspirated, as described previously.\textsuperscript{9} In brief, after measuring the diameter, each follicle was individually aspirated and collected. The volume of FF that initially was aspirated from each follicle was measured by using a 10 mL syringe (Terumo Corporation, Tokyo, Japan) that was connected to an ovum pick-up needle (Kitazato OPU Needle; Kitazato Corporation, Shizuoka, Japan), then was decanted into a 60 mm dish (Corning, Inc., Corning, NY, USA), and the COC was harvested. If the COC was not obtained in the original aspiration, then flushing was performed five times. The COCs were graded according to the following criteria, which are modified criteria of previous studies: grade 1, absent-to-sparse cumulus cells and a dense layer of corona cells with no visible or one visible oocyte, but no clear delineation of the oocyte outline; grade 2, dense cumulus cells and some expansion, with clear delineation of the oocyte outline; and grade 3, expanded cumulus cells and good expansion, with a clear space between the oocyte outline and the corona cell. Initial aspirates containing an abundance of blood were excluded from the analysis. The FF volume was categorized into one of the following six groups: group A, <1.0 mL; group B, 1.0-<2.0 mL; group C, 2.0-<3.0 mL; group D, 3.0-<4.0 mL; group E, 4.0-<5.0 mL; and group F, ≥5.0 mL. Each individually obtained COC was placed into a 5 mL round-bottomed tube (Falcon\textsuperscript{6}; Corning, Inc.) containing 1 mL of HFF99 medium (Fuso Pharmaceutical Industries, Osaka, Japan) with 10% serum protein substitute (Kitazato Corporation) and then was cultured for 2-3 hours until denudation. To prevent an over- or underestimation of the ellipsoid follicular diameter by two-dimensional transvaginal ultrasonography, the FF volume was estimated carefully.\textsuperscript{9}

2.3 Intracytoplasmic sperm injection

The oocytes were separated from the cumulus cells by using .05% hyaluronidase (Sigma-Aldrich Corporation, St. Louis, MO, USA) that was dissolved in the modified human tubal fluid (mHTF) medium (Kitazato Corporation) by pipetting. After denudation, the MII oocytes were cultured for ≥1 hour.\textsuperscript{20} Several spermatozoa were picked up, washed with a 5 μL drop of 5% polyvinylpyrrolidone (Irvine Scientific, Santa Ana, CA, USA) that was dissolved in mHTF with 10% serum protein substitute, and immobilized. The oocytes were inseminated through ICSI by using injection pipettes (inner diameter: 4.7 μm; outer diameter: 6.0 μm; Cook Medical, Bloomington, IN, USA).\textsuperscript{21} The level of oolemma stretchability was evaluated, as described previously.\textsuperscript{11} In brief, the oolemma stretchability during ICSI was evaluated by using an additional mechanical stimulus for oolemma penetration; that is, with or without aspiration (high vs low stretchability, respectively). To ensure objectivity,
Table 1: Relationship between the volume of follicular fluid that was aspirated from individual follicles and the rates of oocyte retrieval and maturity

| Variable                      | Group A (<1.0) | Group B (1.0-<2.0) | Group C (2.0-<3.0) | Group D (3.0-<4.0) | Group E (4.0-<5.0) | Group F (≥5.0) | P-value |
|-------------------------------|----------------|--------------------|--------------------|--------------------|--------------------|----------------|---------|
| No. of aspirated follicles    | 128            | 154                | 170                | 123                | 100                | 76             | -       |
| No. of retrieved oocytes      | 69 (53.9)**    | 97 (63.0)          | 117 (68.8)         | 90 (73.2)          | 78 (78.0)*         | 55 (72.4)     | .001    |
| MII oocytes                   | 48 (69.6)*     | 77 (79.4)          | 102 (87.2)         | 78 (86.7)          | 62 (79.5)          | 48 (87.3)     | .028    |
| Immature oocytes              | 16 (23.2)**    | 14 (14.4)*         | 6 (5.1)            | 4 (4.4)            | 4 (5.1)            | 2 (3.6)       | <.001   |
| Degenerated oocytes           | 5 (7.2)        | 6 (6.2)            | 9 (7.7)            | 8 (8.9)            | 12 (15.4)          | 5 (9.1)       | .372    |

Values are presented as N (%). The number of retrieved oocytes and metaphase II, immature (MII and germinal vesicle), and degenerated oocytes were evaluated by using the chi-square test and residual analysis. A P-value of <.05 was considered to be statistically significant.

*P < .05 and **P < .01.

The oolemma stretchability was determined, regardless of the distance between the penetration point of the oolemma and the edge of the injection pipette during aspiration. The following morning, oocyte fertilization and degeneration were confirmed by the presence of pronuclei (PN) and polar bodies (PBs). The fertilized eggs were individually cultured (CO2 of 6% and O2 of 5% at 37°C) in a 20 μL drop of Global total medium (LifeGlobal Group LLC, Guilford, CT, USA) and covered with light mineral oil (Oil for Embryo Culture; Irvine Scientific) on a 60 mm dish until day 6, until the embryo was cryopreserved on day 3, or until a fresh embryo transfer was performed. The medium was changed on days 1, 3, and 5.

2.4 | Statistical analysis

The mean age of the patients was evaluated by using the Tukey-Kramer post-hoc test and is presented as the mean ± standard deviation. The rates of oocyte retrieval, oocyte maturity, COC grade, and oocyte stretchability were evaluated by using the chi-square test and residual analysis for multiple comparisons. The rates of fertilization, embryo cleavage, ≥7 cells on day 3, and blastocyst development were evaluated by using the chi-square test between two groups. A P-value of <.05 was considered to be statistically significant.

3 | RESULTS

The follicular diameter was correlated with the FF volume that was aspirated from individual follicles (Spearman's rank-order correlation coefficient: .8421, P < .001; data not shown). The rate of oocyte retrieval was significantly lower in group A than in the other groups (53.9% [69/128] vs 63.0%-78.0% [97/154-78/100]; P < .01). The rate of MII oocyte retrieval was significantly lower in group A than in the other groups (69.6% [48/69] vs 79.4%-87.3% [77/97–48/55]; P < .05). The rate of immature oocyte retrieval was significantly higher in groups A and B than in the other groups (14.4%-23.2% [14/97-16/69] vs 3.6%-5.1% [2/55-6/117]; P < .01). There was no significant difference in the oocyte degeneration rates among all groups (Table 1).

The level of oolemma stretchability was significantly lower in group A than in the other groups during ICSI (18.8% [9/48] vs 3.8%-6.5% [3/78-5/77]; P = .022; Table 2). The rates of the COC grades, including the MII oocytes, were not significantly different among all high and low oolemma stretchability groups (Table 2). The rate of oocytes with two PN was significantly lower in group A than that in group C (66.7% [32/48] vs 81.4% [83/102]; P = .0470) and the tendency was lower in group A than in groups B and F (vs 80.5% [62/77] and 83.3% [40/48]; P = .0811 and P = .0593, respectively; Table 3).

There were no significant differences in the rates of oocytes with one PN, oocytes with multiple PN, unfertilized oocytes, and degenerated oocytes after ICSI among all groups (Table 3). Moreover, regarding embryonic development, there were no significant differences in the rates of embryo cleavage and ≥7 cells on day 3 after ICSI among all groups (Table 4). However, the rate of blastocyst development after ICSI was significantly lower in group A than that in group D (40% [8/20] vs 68.4% [26/38]; P = .0367; Table 4). A relationship was noted between the FF volume and MII oolemma stretchability, normal fertilization, and blastocyst development.

4 | DISCUSSION

This study's results demonstrated that the GnRH agonist protocol-derived MII oolemma from a low FF volume has low stretchability, suggesting that the FF volume of individual follicles potentially was associated with the MII oolemma stretchability.

These results showed that the rate of oocyte retrieval from <1.0 mL of FF was low. Even if the COC was obtained, the rate of mature oocyte retrieval was low and that of immature oocyte retrieval was high. Several previous studies have reported that the oocyte maturity rate is associated with the follicular volume/diameter, which is consistent with the current findings.
The incidence of low oolemma stretchability significantly increased when the aspirated FF volume was <1.0 mL during ICSI. This finding is consistent with that of the GnRH antagonist protocol-derived MII oocytes in the authors’ previous study. Accordingly, the FF volume of individual follicles is associated with MII oolemma stretchability, regardless of the type of COS protocol. Therefore, these results speculated that the oolemma stretchability was affected by oocyte maturation, rather than by the COS protocol. Then, the focus was on oocyte maturation and it was found that the follicular size/volume is related to oocyte immaturity, which is relatively higher in oocytes from small follicles. During oocyte maturation, microfilaments and microtubules are spatially rearranged in the cortical and subcortical regions of the germinal vesicle oocytes. Microvillous activity increases as MII is approached and during the abstraction of the PBs, which conglomerate in the constriction zone. Moreover, the germinal vesicle oocyte funnel volume following ICSI is significantly less than that of MI and MII oocytes; that is, cytoplasmic viscosity remarkably changes from an aqueous to a more viscous subtype. These studies showed that components of the oolemma or cytoplasm differ, depending on the oocyte stage.

### Table 2

| Variable                                      | Group A (1.0<2.0) | Group B (2.0<3.0) | Group C (3.0<4.0) | Group D (4.0<5.0) | Group E (5.0<6.0) | P-value |
|-----------------------------------------------|------------------|------------------|------------------|------------------|------------------|--------|
| No. of MII oocytes                            |                  |                  |                  |                  |                  |        |
|                                               | 50               | 77               | 102              | 78               | 62               |        |
| Patient age (years)                           | 37.6±4.0         | 37.6±4.0         | 37.3±3.9         | 37.0±4.3         | 37.1±3.9         | .964   |
| High oolemma stretchability                   | 39 (81.3)        | 72 (93.5)        | 97 (95.1)        | 75 (96.2)        | 58 (93.5)        | .022   |
| COC grade                                     |                  |                  |                  |                  |                  |        |
| 1                                             | 16 (41.0)        | 16 (22.2)        | 22 (22.7)        | 26 (34.7)        | 19 (32.8)        | .155   |
| 2                                             | 20 (51.3)        | 46 (63.9)        | 62 (63.9)        | 42 (56.0)        | 30 (51.7)        | .516   |
| 3                                             | 3 (7.7)          | 10 (13.9)        | 13 (13.4)        | 7 (9.3)          | 9 (15.5)         | .692   |
| Low oolemma stretchability                    | 9 (18.8)         | 5 (6.5)          | 5 (4.9)          | 3 (3.8)          | 4 (6.5)          | .022   |
| COC grade                                     |                  |                  |                  |                  |                  |        |
| 1                                             | 3 (33.3)         | 2 (40.0)         | 2 (40.0)         | 0 (0)            | 2 (50.0)         | .806   |
| 2                                             | 5 (55.6)         | 3 (60.0)         | 2 (40.0)         | 2 (66.7)         | 2 (50.0)         | .983   |
| 3                                             | 1 (11.1)         | 0 (0)            | 1 (20.0)         | 1 (33.3)         | 0 (0)            | .642   |

COC, cumulus–oocyte complex; MII, metaphase II.

Values are presented as N (%) or mean ± standard deviation. The mean age of the patients was evaluated by using the Tukey–Kramer post-hoc test. The rates of oolemma stretchability and COC grades were evaluated by using the chi-square test and residual analysis. A P-value of <.05 was considered to be statistically significant.

*P < .05.

### Table 3

| Variable                                      | Group A (1.0<2.0) | Group B (2.0<3.0) | Group C (3.0<4.0) | Group D (4.0<5.0) | Group E (5.0<6.0) | P-value |
|-----------------------------------------------|------------------|------------------|------------------|------------------|------------------|--------|
| No. of MII oocytes                            |                  |                  |                  |                  |                  |        |
|                                               | 48               | 77               | 102              | 78               | 62               |        |
| Oocytes with 2 PNs and 2 PBs                  | 32 (66.7)        | 62 (80.5)        | 83 (81.4)        | 55 (70.5)        | 46 (74.2)        | .022   |
| Oocytes with 1 PN                             | 5 (10.4)         | 5 (6.5)          | 3 (2.9)          | 6 (7.7)          | 4 (16.7)         | .242   |
| Oocytes with multiple PNs                     | 0 (0)            | 1 (1.3)          | 4 (3.9)          | 3 (3.8)          | 2 (3.2)          | 0 (0)  |
| Unfertilized oocytes                          | 8 (16.7)         | 7 (9.1)          | 9 (8.8)          | 12 (15.4)        | 8 (12.9)         | .08    |
| Degenerated oocytes                           | 3 (6.3)          | 2 (2.6)          | 3 (2.9)          | 2 (2.6)          | 2 (3.2)          | 1 (2.1)|

MII, metaphase II; PB, polar body; PN, pronucleus.

Values are presented as N (%). The rates of oocytes with two PNs and two PBs, one PN, or multiple PNs, unfertilized oocytes, and degenerated oocytes were evaluated using the chi-square test for between group A and each group. A P-value of <.05 was considered statistically significant.

*P < .05: significant difference between groups A and C.
and that components of the oocyte drastically change during oocyte maturation. Therefore, it was speculated that even if an oocyte from a small FF volume is in stage MII, differences in the FF volume and microenvironment potentially can affect the rearrangement of the oolemmic or cytoplasmic components during oocyte maturation after hCG injection and that the incidence of low oolemma stretchability is relatively greater in the MII oocytes that are derived from a small FF volume during ICSI.

The FF volume was not associated with the rates of the COC grade, including the MII oocytes. Hence, the impact on low stretchability in this study is unlikely to be related to the COC grades, including the MII oocytes. Meanwhile, the FF volume was associated with the rates of normal fertilization. In addition, although the FF volume was not associated with the rates of embryo cleavage and ≥7 cells on day 3, it was associated with the rate of blastocyst development in this study. The results of normal fertilization and blastocyst development are not consistent with the findings of the authors’ previous GnRH antagonist study. Several studies have reported a relationship between the follicular size/volume and the fertilization rate following ICSI, although there is no consensus on the effect of the FF size/volume on fertilization following ICSI. This study’s data showed that the FF volume had an impact on the fertilization rate. A previous study showed that the fertilization rate of low-quality oocytes, such as with a fragile oolemma, was low. Accordingly, it was speculated that the reduction of the normal fertilization rate in the low FF volume (<1.0 mL) group was associated with an increasing incidence of low oolemma stretchability.

Regarding embryonic development, the follicular size and oocyte developmental factors, such as cleavage, implantation, clinical pregnancy, and live birth, were not closely related and thus were considered to be independent. Moreover, one study reported by time-lapse analysis that the presence of a fragile oolemma does not affect the subsequent fertilization and developmental processes of fragile oocytes. However, this study’s results showed that the FF volume had an impact on the blastocyst development and that the rate of blastocyst development was low in the <1.0 mL group, including more low stretchability oocytes. Accordingly, it was speculated that the reduction of the blastocyst development rate in the <1.0 mL group was associated with the FF volume and an increasing incidence of low oolemma stretchability.

The application of these results is limited because of the retrospective nature of the analysis. The association of the FF volume with the rates of pregnancy and live births also were not analyzed because two embryos or blastocysts frequently were transferred into the uterus in accordance with the recommendations of the Japan Society for Obstetrics and Gynecology.

In conclusion, the FF volume potentially is associated with human MII oolemma stretchability during ICSI, regardless of the type of COS protocol. Moreover, a low FF volume-derived oocyte tended to have low fertilization and blastocyst development abilities. In terms of oolemma stretchability, oocyte maturity, fertilization, and development, ensuring a uniform follicular size during COS is crucial for obtaining good-quality oocytes.

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DISCLOSURES

Conflict of interest: The authors declare no conflict of interest. Human rights statement and informed consent: All the procedures were followed in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all the patients who were included in the study. The procedure was not referred to the Institutional Review Board of Umeda Fertility Clinic because this study was classified as an un invasive, anonymous, and retrospective database study. Animal studies: This article

| Variable                              | Group A (<1.0) | Group B (1.0-<2.0) | Group C (2.0-<3.0) | Group D (3.0-<4.0) | Group E (4.0-<5.0) | Group F (≥5.0) |
|--------------------------------------|---------------|-------------------|-------------------|-------------------|------------------|----------------|
| Oocytes with 2 PN and 2 PBs          | 32            | 62                | 83                | 55                | 46               | 40             |
| Cleavage                             | 31/32 (96.9)  | 61/62 (98.4)      | 81/83 (97.6)      | 55/55 (100.0)     | 46/46 (100.0)    | 38/40 (95.0)   |
| ≥7 cells on day 3                    | 17/31 (54.8)  | 35/61 (57.4)      | 49/81 (60.5)      | 40/55 (72.7)      | 23/46 (50.0)     | 23/38 (60.5)   |
| No. of cultured oocytes until day 6  | 20            | 49                | 61                | 38                | 37               | 28             |
| Blastocysts                          | 8 (40.0)*     | 28 (57.1)         | 37 (60.7)         | 26 (68.4)*        | 22 (59.5)        | 18 (64.3)      |

PB, polar body; PN, pronucleus.
Values are presented as N (%). The rates of cleavage, ≥7 cells on day 3, and blastocyst development were evaluated by using the chi-square test between group A and each group. A P-value of <.05 was considered to be statistically significant.

*P < .05: significant difference between group A and group D.
does not contain any study with animal participants that were performed by any of the authors.

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