J. Mamm. Ova Res. Vol. 41 (1), 35–44, 2024

—Original—

The area of oocyte cytoplasmic shrinkage can be used to predict fertilization of oocytes after ICSI

Keiko Yamaguchi[1, a], Fumiaki Itoi[1*, 4, a], Hiroyuki Honnma[2], Kyouko Fujita[1], Tomomi Seki[3], Utako Fujii[1], Sayako Ikeda[1] and Miho Sano[1]

[1]*Department of Reproduction Center, Komaki City Hospital, Komaki, Aichi 485-8520, Japan*
[2]*Sapporo ART Clinic, Kita-ku, Sapporo, Hokkaido 060-0807, Japan*
[3]*Department of Obstetrics and Gynecology, Nagoya University, Showa-ku Nagoya, Aichi 466-8550, Japan*
[4]*Present affiliation: Nagoya ART Clinic, Nakamura-ku, Nagoya, Aichi 453-0801, Japan*

Abstract: We examined whether oocyte cytoplasmic shrinkage after ICSI can be used for early prediction of fertilization. This was a retrospective study of 164 two pronuclei (2PN) zygotes, 19 abnormal zygotes (11 1PN and 8 ≥3PN) and 26 unfertilized (0PN) oocytes in 120 cycles which were observed using a time-lapse system. Image collection began 30 min after ICSI, and measurements of oocyte cytoplasmic area and diameter were made 13 times over the course of 5.3 h. Following ICSI, cytoplasmic areas shrank significantly more between <0.5–0.9 h (98.8 ± 1.4%) (mean ± SD) and <4.9–5.3 h (92.2 ± 2.6%) in 2PN zygotes than they did in 0PN oocytes (99.8 ± 1.2%, 97.7±1.9%; $P < 0.01$). Similar to 2PN zygotes, the shrinkage rates of the cytoplasmic areas declined over time in both 1PN and ≥3PN zygotes. However, the normality or abnormality of fertilization could not be distinguished by the measurement. The area under the ROC curve and optimal cutoff value for the prediction of fertilization were 0.947 and 97.120%, respectively, at <2.9–3.3 h after ICSI. Oocyte cytoplasmic shrinkage had no significant effect on embryo development. Measurement of oocyte cytoplasmic shrinkage after ICSI can confirm fertilization within 5.3 h with a fairly high accuracy and can be a useful criterion for fertilization prediction.
Key words: ICSI, fertilization prediction, unfertilized oocyte, oocyte cytoplasmic shrinkage

Received: July 20, 2023
Accepted: September 21, 2023
*To whom correspondence should be addressed.
[a]These authors contributed equally to this work.
e-mail: itofumi1980@gmail.com

Introduction

Intracytoplasmic sperm injection (ICSI) has become an important method of fertilization for male factor infertility in human-assisted reproductive technology [1]. In some cases, however, the fertilization rate is low or there is no fertilization at all, even after ICSI. These are called partial or complete fertilization failures and account for 1%–5% of total ICSI cycles [2, 3]. Artificial oocyte activation (AOA) after ICSI is effective in these cases of fertilization failure [4, 5]. However, several confirming cycles are necessary to determine whether the patients have fertilization failure, which causes emotional distress and is a financial burden on the patients. The cycle is canceled and the unfertilized (0PN) oocytes are discarded only if there were 0PN oocytes after ICSI, especially in cases where the number of oocytes collected is small. When it has been determined that the oocytes were not fertilized, the current practice is to perform AOA of oocytes on the day after ICSI in an effort to obtain two pronuclei (2PN) zygotes [6–10]. Although this approach can produce 2PN zygotes, their subsequent development rate is very low [6–8], and there is currently only a single reported case of a live birth [9]. In recent years, 2PN zygotes have been obtained through rescue AOA (rAOA) of 0PN oocytes on the day of ICSI, with fertilization judged on the basis of second polar body extrusion, and there have been births after embryo transfer similar to normal ICSI [11].

It is well established that the cytoplasm in human oocytes shrinks upon fertilization [12, 13]. Using time-lapse video cinematography, Payne *et al.* [12] observed that, after ICSI, the diameter of 2PN zygotes shrank from

111.6 ± 3.6 μm at injection to 106.0 ± 4.6 μm 17 h post-injection. Subsequently, Liu *et al.* [13] used a time-lapse system to measure and compare the cytoplasmic areas of 2PN zygotes and 0PN oocytes every hour for up to 9 h after ICSI. They found that within 3 h, the cytoplasmic area of 2PN zygotes shrank significantly compared to 0PN oocytes [13]. However, there are as yet no reports on the cytoplasmic shrinkage of abnormal zygotes (1PN and ≥3PN) or the relationship between cytoplasmic shrinkage and embryogenesis.

The present study retrospectively examined time-lapse images to determine whether fertilization can be effectively predicted within a short period on the basis of oocyte cytoplasmic shrinkage using measurements of the cytoplasmic area and diameter for 2PN zygotes, abnormal zygotes (1PN and ≥3PN), and 0PN oocytes after ICSI. In addition, we assessed the relationship between embryonic development and the oocyte cytoplasmic shrinkage rate.

Materials and Methods

Patients

This retrospective cohort study included 87 patients who underwent 120 ICSI treatment cycles (Table 1) at the Department of Reproduction Center between June 2018 and December 2020. In this study, although ICSI was performed on a total of 590 oocytes in 120 treatment cycles, only the 209 oocytes placed in the time-lapse system within 30 min after ICSI were examined. Moreover, we were unable to measure cytoplasmic shrinkage in oocytes with poor morphology similar to previously reported [13]. ICSI was performed for patients without male factor infertility, the inability to obtain sperm by the density-gradient centrifugation technique and swim-up method, repeated fertilization failures, and older women. Oocytes artificially activated after ICSI were excluded from this study. Our center does not use donor oocytes. All patients provided consent to all treatment procedures and agreed to the anonymous use of their data for studies. This study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional review board (approval no. 201009).

Ovarian Stimulation, ICSI, Fertilization Evaluation and Embryo culture

Parts of the protocols for ovarian stimulation, ICSI, and fertilization evaluation shown in Table 1 and were previously described by Itoi *et al.* [14]. Briefly, the ovarian stimulation protocols were performed using a GnRH antagonist (Cetrotide; Merck Biopharma Co., Ltd., Tokyo, Japan) and a GnRH agonist (Nafarelin acetate; Nafarelil, Fuji Pharma Co., Ltd., Tokyo, Japan) in a short protocol, or by using chlormadinone acetate (Lutoral; Shionogi and Co., Ltd., Osaka, Japan) in a PPOS protocol [15] with recombinant FSH (Gonal-F®, Merck Biopharma Co., Ltd.) and/or hMG (Ferring Pharmaceuticals, Tokyo, Japan) and clomiphene citrate (Serophene; Merck Biopharma Co., Ltd.). Recombinant HCG (rHCG; Ovidrel®, Merck Biopharma Co., Ltd.) or GnRH agonist was administered when the leading follicles averaged 18 mm in diameter. Ultrasound-guided transvaginal oocyte retrieval was performed 37 h after rHCG administration or 36 h after GnRH agonist administration. Oocytes were fertilized using the ICSI technique within 2–7 h after retrieval. For the ICSI procedure, an immobilized sperm was injected into the oocyte using an injection pipette (Sankyo Medic, Shizuoka, Japan). Fertilization was assessed 18–19 h after ICSI. The zygotes were transferred to CSCM-NXC (FUJIFILM Irvine Scientific, Osaka, Japan) and cultured in a time-lapse system (EmbryoScope™; Vitrolife, Stockholm, Sweden) until day 5 or 6 at 37°C under 6% CO_2, 5% O_2 and 89% N_2. Embryo quality was evaluated on day 3 using the grading system of Veeck [16]. Blastocysts were evaluated using the grading system of Schoolcraft *et al.* [17].

Time-Lapse System

Using an EmbryoScope™, 2PN zygotes, abnormal zygotes (1PN and ≥3PN) and 0PN oocytes were observed following ICSI. The EmbryoSlide designed for culture in EmbryoScope™ was then set, and 11 images along the z-axis were captured at 20 min intervals, resulting in the collection of about 165 images up to 5.3 h, and about 4,000 images up to the blastocyst stage.

Measurement of Oocyte Cytoplasmic Size

Oocyte cytoplasmic areas and diameters were measured by one embryologist using the *Ellipse* and *Line* functions of EmbryoViewer® software [13] (Fig. 1A). This study analyzed 164 2PN zygotes, 19 abnormal zygotes (11 1PN and 8 ≥3PN) and 26 0PN oocytes that were observed up to the blastocyst stage in an EmbryoScope™ from within 30 min after ICSI. The areas and diameters for detection of oocyte cytoplasmic shrinkage were measured over the course of 5.3 h divided into 13 intervals (0.1–0.5, <0.5–0.9, <0.9–1.3, <1.3–1.7, <1.7–2.1, <2.1–2.5, <2.5–2.9, <2.9–3.3, <3.3–3.7, <3.7–4.1, <4.1–4.5, <4.5–4.9, and <4.9–5.3 h) beginning at the time observation was started in the EmbryoScope™ (Fig. 1B). The cytoplasmic shrinkage rate was calculated by dividing the area or diameter at each time by the area or diameter

Table 1. Patient characteristics and embryological outcomes in ICSI cycles

No. of cycles	120
Female age (years)	36.9 ± 4.4
Mean no. of cycles	1.4 ± 0.7
Cause of infertility	
Female factor (%)	43 (36)
Male factor (%)	31 (26)
Female and male factor (%)	22 (18)
Unexplained (%)	24 (20)
Ovarian stimulation protocol	
Antagonist (%)	23 (19)
Short agonist (%)	8 (7)
PPOS (%)	17 (14)
Mild stimulation (%)	72 (60)
Duration of stimulation (days)	10.7 ± 3.4
Total dose of gonadotrophins (IU/mL)	1,341.0 ± 1,285.1
Serum estradiol concentration (pg/mL)	1,869.7 ± 1,534.1
Ovarian retrieval	
Mean no. of retrieved oocytes	6.7 ± 7.2
Mean no. of MII oocytes	5.6 ± 6.4
Fertilization	
Percent of 2PN	81.6 ± 26.7
Percent of abnormal zygotes (1PN and ≥3PN)	3.9 ± 10.5
Percent of 0PN	0.5 ± 1.1
Percent of degeneration	0.3 ± 0.9
No. of analyzed oocytes	
No. of 2PN	164
No. of abnormal zygotes (1PN and ≥3PN)	19 (11 1PN and 8 ≥3PN)
No. of 0PN	26

Data are shown as means ± SD. *ICSI* intracytoplasmic sperm injection, *PPOS* progestin prime ovarian stimulation protocol, *serum estradiol concentration* serum estradiol concentration on the day of HCG administration, *MII* metaphase II, *analyzed oocytes* injected oocytes using, *2PN* two pronuclei, *1PN* single pronucleus, *≥3PN* three and multiple pronuclei, *0PN* unfertilized oocyte.

at 0.1–0.5h. The reason for this was the analyzed 2PN zygotes had an average polar body release time of 2.8 h, and the polar body was released within 5 h after ICSI at the latest. The relationship between the cytoplasmic shrinkage rate and the position of the first polar body, juxtaposed (Fig. 1C) or overlapping (Figs. 1D and 1E) in MII oocytes, was also evaluated.

Statistical Analysis

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [18]. The differences between the relative oocyte cytoplasmic sizes were compared using the Mann-Whitney U test or Kruskal-Wallis test. The χ^2-test was used to evaluate the significance of differences between the relative sizes (%). Values of $P < 0.05$ were considered significant. Cutoff values for assessing whether fertilization had occurred, based on oocyte cytoplasmic sizes in 2PN zygotes and 0PN oocytes, were determined from receiver-operating characteristic (ROC) curves.

Results

Oocyte Cytoplasmic Shrinkage Rates in 2PN Zygotes and 0PN Oocytes

The rate at which the oocyte cytoplasmic area shrank in 2PN zygotes significantly declined over time after ICSI (cytoplasmic reduction <0.5–0.9 h: 98.8 ± 1.4% (mean ± SD) to <4.9–5.3 h: 92.2 ± 2.6%), compared to 0PN oocytes (cytoplasmic reduction <0.5–0.9 h: 99.8 ± 1.2% to <4.9–5.3 h: 97.7 ± 1.9%) ($P < 0.01$; Figs. 2A and 2C). Moreover, at each time interval from <0.5–0.9 h to <2.9–3.3 h, the oocyte cytoplasmic area of 2PN zygotes was significantly smaller than in the previous interval (*P*

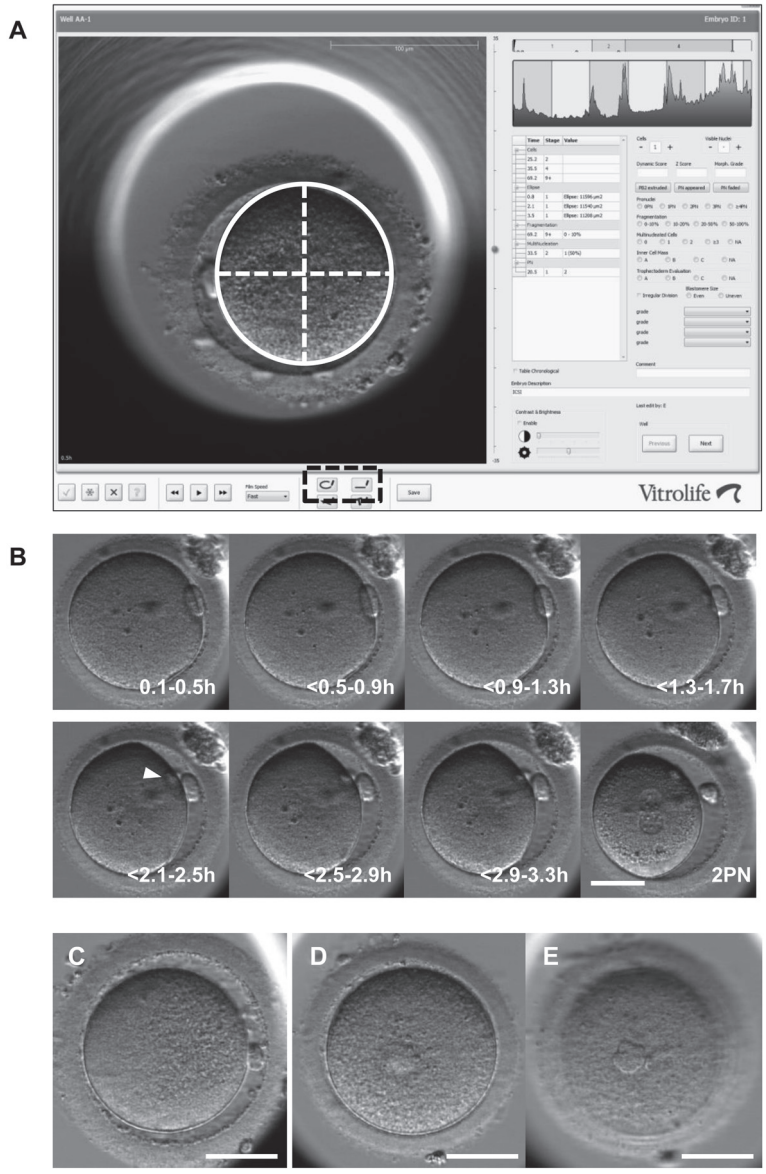

Fig. 1. Measurement of oocyte cytoplasmic area and diameter after ICSI. **A** Screenshot of EmbryoViewer® software and the method for measuring the oocyte cytoplasmic area (*white circle*) and diameter (*white dotted lines*) using the *Ellipse* and *Line* functions (*black dotted square*). **B** Images showing oocyte cytoplasmic shrinkage in fertilized zygotes after ICSI as well as extrusion of the second polar body (*arrowhead*) at <2.1–2.5 h. **C**–**E** Images of the position of the first polar body in MII oocytes: juxtaposed (**C**) and overlapping (**D** and **E**). *Scale bars* are 50 μm.

< 0.01, <0.5–0.9 to <2.5–2.9 h; $P < 0.05$, <2.5–2.9 to <2.9–3.3 h). Although the cytoplasmic areas of 0PN oocytes significantly declined from <0.5–0.9 to <0.9–1.3 h ($P < 0.05$), no significant shrinkage was observed from that time point onward (Fig. 2C).

The pattern of reductions in the diameters of the cytoplasm during the 5.3 h observation period was similar to the pattern of reductions in the cytoplasmic areas of both the 2PN zygotes (diameter reduction 99.4 ± 0.8% to 95.9 ± 1.5%) (mean ± SD) and 0PN oocytes (diameter reduction 99.8 ± 0.7% to 98.8 ± 1.0%) (Figs. 2B and 2D). However, because differences in the time-dependent chang-

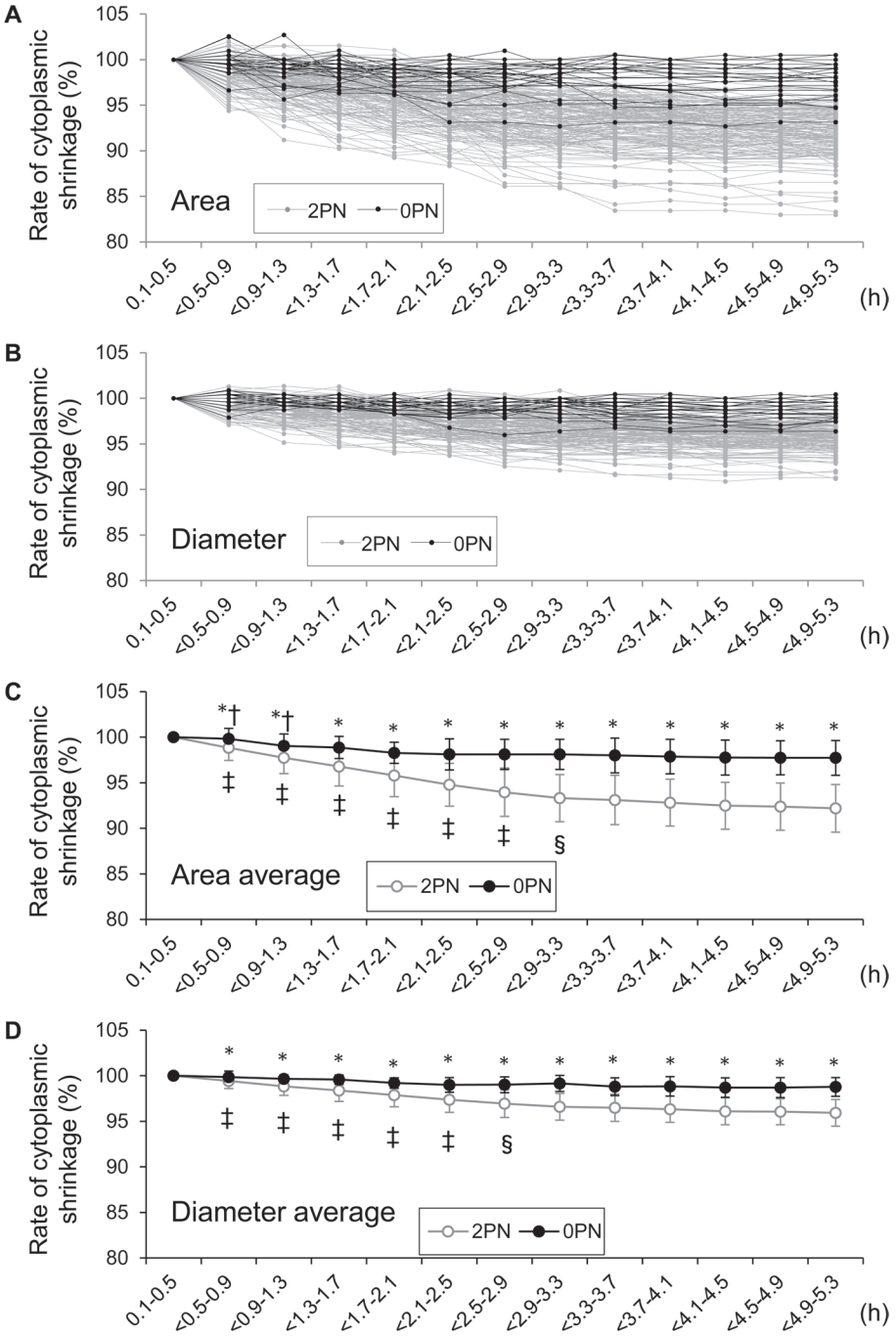

Fig. 2. Time-dependent changes in the relative cytoplasmic area and diameter in 2PN zygotes and 0PN oocytes after ICSI. **A and B** Graphs showing the time-dependent changes in relative cytoplasmic areas (**A**) and diameters (**B**) in 2PN zygotes (*light gray*) and 0PN oocytes (*black*) after ICSI. **C and D** Graphs showing the time-dependent changes in average relative cytoplasmic area (**C**) and diameter (**D**) of 2PN zygotes (*light gray*) and 0PN oocytes (*black*) after ICSI. *$P < 0.01$ 2PN vs. 0PN, $^{\dagger}P < 0.05$ vs. 0PN previous time interval, $^{\ddagger}P < 0.01$ 2PN vs. previous time interval, $^{\S}P < 0.05$ vs. 2PN previous time interval.

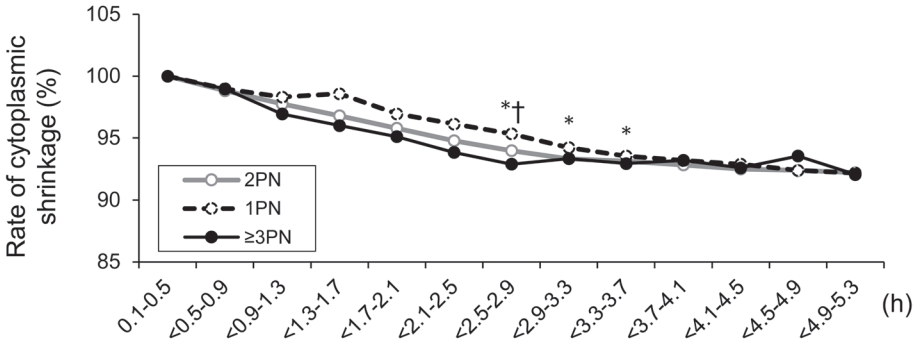

Fig. 3. Time-dependent changes in the relative cytoplasmic area of abnormal zygotes (1PN and ≥3PN) after ICSI. The graph shows the average relative cytoplasmic areas in 2PN zygotes (*gray*), 1PN zygotes (*dashed black*) ≥3PN zygotes (*black*). $^{*\dagger}P < 0.05$ 1PN (<0.5–0.9 h) vs. (<2.1–2.5 h, <2.5–2.9 h and <2.9–3.3 h). $^{\dagger}P < 0.05$ 2PN (<0.5–0.9 h) vs. (<2.1–2.5 h).

es in oocyte cytoplasmic area between 2PN zygotes and 0PN oocytes were more pronounced, the cytoplasmic areas were used for subsequent analyses.

Oocyte Cytoplasmic Shrinkage Rates in Abnormal Zygotes (1PN and ≥3PN)

As in 2PN zygotes, the shrinkage rates of the cytoplasmic areas declined over time after ICSI in both 1PN zygotes (cytoplasmic reduction <0.5–0.9 h: 99.0 ± 2.3% vs. <4.9–5.3 h: 92.2 ± 2.4%) (mean ± SD) and ≥3PN zygotes (cytoplasmic reduction <0.5–0.9 h: 99.0 ± 3.4% vs. <4.9–5.3 h: 92.1 ± 7.1%) (Fig. 3). The cytoplasmic shrinkage rates of 1PN and ≥3PN zygotes were not significantly different from that of 2PN zygotes.

Relationship Between Oocyte Cytoplasmic Shrinkage Rates and the Position of the First Polar Body and the Extrusion Time of the Second Polar Body

Whether the position of the first polar body was juxtaposing (Fig. 1C) or overlapping (Figs. 1D and 1E) did not significantly affect the rate of cytoplasmic shrinkage in 2PN zygotes (Fig. 4A). The reason for this was the average release time of the second polar body in 2PN zygotes was 2.8 h. The oocyte cytoplasmic shrinkage rate was also compared between the observation periods ≤2.8 h and >2.8 h. The decline in oocyte cytoplasmic area between <1.7–2.1 h (95.3 ± 2.5%) (mean ± SD) and <2.5–2.9 h (93.5 ± 2.7%) was significantly greater than at times >2.8 h (96.4 ± 1.9%, 94.5 ± 2.5%; $P < 0.05$). However, cytoplasmic shrinkage of 2PN zygotes was significantly greater than that of 0PN oocytes during both the early and later periods ($P < 0.01$; Fig. 4B).

ROC Curve Analysis of the Optimum Cutoff Value and Time for Prediction of Fertilization

ROC curve analysis was used to determine the optimum time for fertilization prediction based on the oocyte cytoplasmic shrinkage rate of each time interval. The AUC, 95% confidence interval, and the cutoff value were calculated to be 0.947, 0.895–0.999, and 97.120%, respectively, at <2.9–3.3 h after ICSI (Table 2).

Relationship between the Oocyte Cytoplasmic Shrinkage Rate and Embryo Development

Embryological outcomes were compared between oocytes exhibiting average cytoplasmic shrinkage to ≤93.2% or >93.2% at 3.0–3.3 h after ICSI (Table 3). The average cytoplasmic shrinkage rate of the 164 2PNs analyzed was 93.3%, therefore, the comparison was made between ≤93.2% and >93.2%. Although the embryonic development rate on day 3 was significantly higher in the >93.2% (61.8%) than the ≤93.2% group (42.7%), there was no significant difference between the rates of blastocyst formation (52.8% vs. 46.7%) or the rates of good quality blastocysts (32.6% vs. 25.3%).

Relationship between the Observation Start Time and Oocyte Cytoplasmic Shrinkage Rates after ICSI

We shifted the start time of observation of 164 2PN zygotes and 26 0PN oocytes placed in the time-lapse system within 30 min. When observation using the time-lapse system was started at around 1.3 h or later after ICSI, the tendency was the same as when observation started within <0.5–0.9 h after ICSI (Fig. 2C and Figs. 5A and 5B). However, the difference in oocyte cytoplasmic shrinkage between 2PN zygotes (cytoplasmic reduction <1.7–2.1 h: 99.0 ± 1.2% to <4.9–5.3 h: 95.3 ± 2.4%) (mean

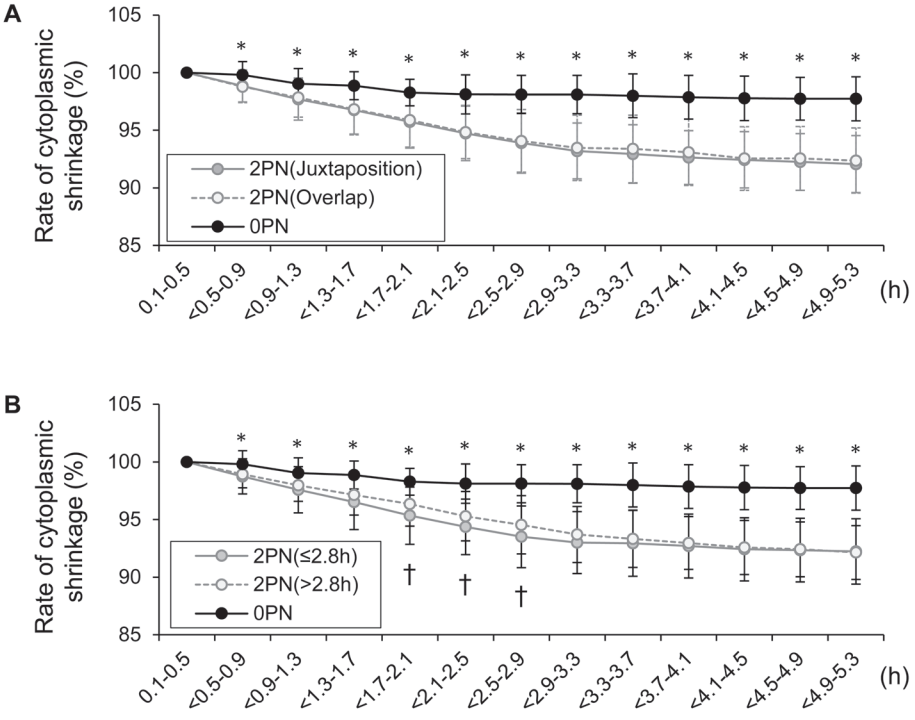

Fig. 4. Time-dependent changes in the relative cytoplasmic areas of 2 PN zygotes and 0PN oocytes and their relation to the position of the first polar body and the time of second polar body extrusion. **A** Average relative cytoplasmic areas of 2PN zygotes (juxtaposition *gray*, overlap *dashed light gray*) and 0PN oocytes (*black*) showing the lack of effect of the position of the first polar body. **B** Average relative cytoplasmic areas of 2PN zygotes (≤2.8 h *gray*, >2.8 h *dashed light gray*) and 0PN oocytes (*black*) showing the impact of extrusion time of the second polar body. **P* < 0.01 2PN vs. 0PN, †*P* < 0.05 2PN (≤2.8 h) vs. (>2.8 h).

Table 2. Cutoff value using ROC curve for the optimum time of fertilization prediction

Time section after ICSI (hours)	Cutoff value	AUC	95% CI
<0.5–0.9	99.435	0.710	0.618–0.805
<0.9–1.3	98.568	0.731	0.631–0.832
<1.3–1.7	98.565	0.804	0.724–0.884
<1.7–2.1	97.983	0.841	0.771–0.910
<2.1–2.5	97.581	0.890	0.815–0.965
<2.5–2.9	96.583	0.920	0.864–0.976
<2.9–3.3	97.120	0.947	0.895–0.999
<3.3–3.7	97.027	0.941	0.892–0.990
<3.7–4.1	96.556	0.948	0.903–0.994
<4.1–4.5	95.049	0.956	0.912–1.000
<4.5–4.9	95.049	0.961	0.925–0.998
<4.9–5.3	94.710	0.963	0.929–0.997

ROC curve receiver operator characteristics curve, *ICSI* intracytoplasmic sperm injection, *AUC* area under the curve, *CI* confidence interval.

Table 3. Relationship between the average of the oocytes cytoplasmic shrinkage rate at 3.0–3.3 h post ICSI and embryo development

Area of oocytes cytoplasmic shrinkage	≤93.2%	>93.2%
Female age (years)	37.7 ± 3.9	36.7 ± 4.2
Time of 2nd PB extrusion (hours)	2.6 ± 0.6 [a]	2.9 ± 0.7 [b]
No. of embryos	75	89
Rate of good quality embryos at day 3 (%)	32 (42.7) [a]	55 (61.8) [b]
Rate of blastocyst formation (%)	35 (46.7)	47 (52.8)
Rate of good quality blastocyst (%)	19 (25.3)	29 (32.6)

Data are shown as means ± SD for each group, and the statistical significance of differences between group characteristics was assessed using the Mann-Whitney U-test. The χ^2-test was used to evaluate the significance of differences between the proportions. a vs. b: values with different superscript letters are significantly different (P<0.05). *PB* polar body, *Good-quality embryos at day 3* 7–9 cells with ≤10% fragmentation, *Good-quality blastocysts* blastocyst expansion grade 3≤, inner cell mass grade B≤, trophectoderm grade B≤.

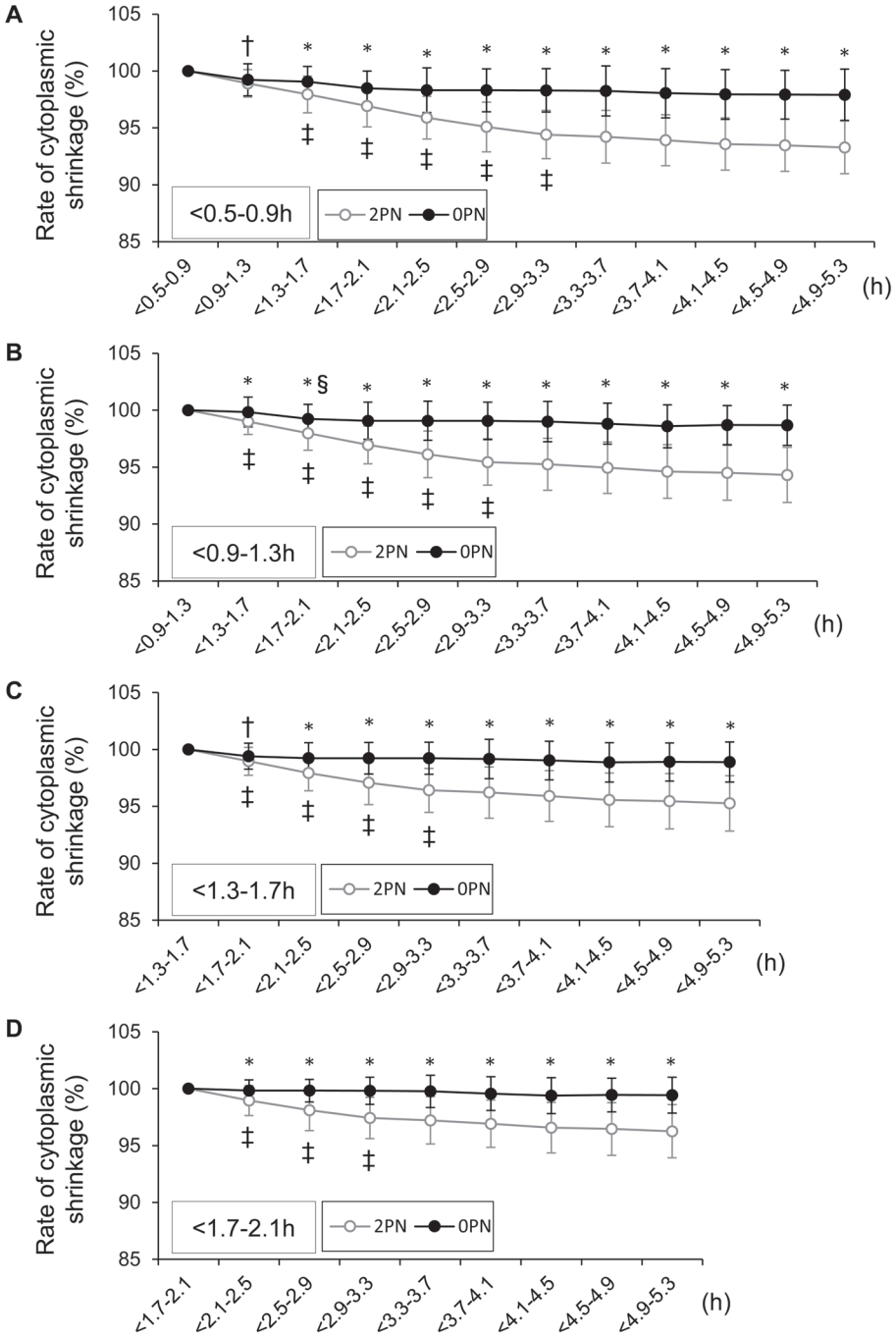

Fig. 5. **A–D** Time-dependent changes in relative cytoplasmic area of 2PN zygotes and 0PN oocytes showing the effect of observation start time. *$P < 0.01$ 2PN vs. 0PN, †$P < 0.01$ 0PN vs. previous time interval, ‡$P < 0.01$ 2PN vs. previous time interval, §$P < 0.05$ 0PN vs. previous time interval.

± SD) and 0PN oocytes (cytoplasmic reduction <1.7–2.1 h: 99.4 ± 1.1% to <4.9–5.3 h: 98.9 ± 1.8%) was smaller in the intervals when observation was started more than <1.3–1.7 h after ICSI (Figs. 5C and 5D).

Discussion

This study demonstrated that measurements of oocyte cytoplasmic shrinkage after ICSI can be used to confirm

early fertilization with a fairly high accuracy. In particular, the ROC curve analysis showed that fertilization can be determined with very high accuracy within <2.9–3.3 h after ICSI. Our results suggest that reduction in oocyte cytoplasmic area could be used as one of the criteria for performing rAOA.

Although AOA reportedly improves fertilization rates and clinical outcomes in cases of partial or complete fertilization failure, it is usually performed immediately or within about 30 min after ICSI [4, 19]. Moreover, earlier reports have suggested that rates of activation and development are very low with AOA in oocytes on the day after ICSI [6–8]. More recently, Economou *et al.* reported that AOA induced in 0PN oocytes on the day after ICSI using calcium ionophores with granulocyte-macrophage colony-stimulating factor improved embryo development [10]. At present, however, only one case of a live birth after AOA on the day after ICSI has been reported [9]. Shibahara *et al.* reported performing early rAOA on 0PN oocytes on the day of ICSI and fertilization prediction based on second polar body extrusion [11]. Although the rate of 2PN zygotes and embryo development to blastocysts were significantly lower than with spontaneously activated oocytes, no difference in clinical outcomes was noted [11]. Their technique would be very useful for 0PN oocytes on the day of ICSI. However, rAOA was performed late in the protocol (from 17:30 to 18:30; ≥2.5 and <6 h after ICSI) and entailed observing the spindle body orientation and the second polar body [11]. Accordingly, it would take up to 6 or more hours from confirming the second polar body extrusion at the scheduled time of 17:30 to 18:30 to performing rAOA. Thus, the fertilization prediction method described in the present study using the phenomenon of oocyte cytoplasmic shrinkage may be advantageous because it can more accurately confirm over time whether or not fertilization has taken place.

A few reports have noted oocyte cytoplasm shrinkage after fertilization. Payne *et al.* detected a significant reduction in oocyte cytoplasmic diameter measured at two time points after ICSI [12]. Liu *et al.* obtained images using a time-lapse system and then used an attached tool to measure the oocyte cytoplasmic area every hour up to 9 h after ICSI [13]. However, the details of the imaging start time were not reported in their study. Consequently, the cytoplasm in some oocytes may have already shrunk when imaging was started. The present study has demonstrated the possibility of predicting fertilization <2.9–3.3 h after ICSI by placing oocytes in a time-lapse system within 0.5 h after ICSI and capturing images every 20 min (Figs. 2C and 2D). It is also the first study to examine cytoplasm shrinkage in abnormal zygotes (1PN and ≥3PN). The finding that cytoplasmic shrinkage of both 1PN and ≥3PN zygotes has a similar tendency to that of 2PN zygotes indicates that the oocyte cytoplasm shrinks when fertilized, irrespective of the fertilization status (Fig. 3). This is thought to be because biological phenomena such as calcium oscillations [20] after sperm injection occur as in normal fertilization, regardless of the fertilization status. In addition, the present study showed that the position of the first polar body has no influence on cytoplasmic shrinkage after oocytes have been placed in an EmbryoScope™ (Fig. 4A). Furthermore, analysis of the imaging start time suggests that for fertilization prediction, oocytes should be placed into an EmbryoScope™ within 1.3 h after ICSI (Fig. 5).

There have been no previous reports on the relation between the cytoplasmic shrinkage rate and embryo development. The results of the present study suggest that cytoplasmic shrinkage are not associated with the rates of blastocyst formation or good blastocysts. These results are similar to those of a previous study [13] which reported that although the volume regulation system changed, depending on the osmolyte transporting systems at each stage, it did not affect embryo development.

Because oocytes need to be placed in a time-lapse observation system within 1.3 h after ICSI, fertilization prediction based on oocyte cytoplasmic shrinkage may need to be restricted to patients from whom only a small number of oocytes have been collected. However, because the rates of cumulative pregnancy and live birth are low in cases with a small number of oocytes [21, 22], we consider that rAOA should be performed only in cases when only a small number of oocytes is available, in order to obtain as many fertilized oocytes as possible, in order to create the opportunity to increase the number of cryopreserved embryos, and consequent opportunities for embryo transfer, and pregnancies. Unfortunately, fertilization failure occurs even in cases when a large number of oocytes have been collected, but it is thought that this can be resolved by performing ICSI with multiple embryologists.

To conclude, the present results suggest that measuring oocyte cytoplasmic area may be a more accurate means of assessing cytoplasmic shrinkage than measuring cytoplasmic diameter because oocyte diameter changes are limited to several micrometers. Consequently, measurement errors are likely to have a greater impact when measuring cytoplasmic diameter than when measuring the area and the possibility of measurement errors must be taken into consideration because these measurements are made manually using a tool attached to an EmbryoScope™. In the future, to perform rAOA for

0PN oocytes quickly it will be necessary to develop tools that can accurately measure oocytes' cytoplasmic area on the basis of the images captured in the time-lapse system.

Acknowledgments

The authors thank the staff of the Department of Reproduction Center in Komaki City Hospital for their assistance in preparing this manuscript and for their expert technical help.

References

1) Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992): Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. The Lancet, 340, 17–18.
2) Kashir, J., Heindryckx, B., Jones, C., de Sutter, P., Parrington, J. and Coward, K. (2010): Oocyte activation, phospholipase C zeta and human infertility. Hum. Reprod. Update, 16, 690–703.
3) Long, R., Wang, M., Yang, Q. Y., Hu, S. Q., Zhu, L. X. and Jin, L. (2020): Risk of birth defects in children conceived by artificial oocyte activation and intracytoplasmic sperm injection: a meta-analysis. Reprod. Biol. Endocrinol., 18, 1–9.
4) Ebner, T., Oppelt, P., Wober, M., Staples, P., Mayer, R.B., Sonnleitner, U., Bulfon-Vogl, S., Gruber, I., Haid, A.E. and Shebl, O. (2015): Treatment with Ca2+ ionophore improves embryo development and outcome in cases with previous developmental problems: A prospective multicenter study. Hum. Reprod., 30, 97–102.
5) Lv, M., Zhang, D., He, X., Chen, B., Li, Q., Ding, D., Hao, Y., Xue, R., Ji, D., Zou, W., Zou, H., Liu, Y., Wang, J., Wei, Z., Zhou, P., Cao, Y. and Zhang, Z. (2020): Artificial oocyte activation to improve reproductive outcomes in couples with various causes of infertility: a retrospective cohort study. Reprod. BioMed. Online, 40, 501–509.
6) Zhang, J., Wang, C.W., Blaszcyzk, A., Grifo, J.A., Ozil, J., Haberman, E., Adler, A. and Krey, L.C. (1999): Electrical activation and in vitro development of human oocytes that fail to fertilize after intracytoplasmic sperm injection. Fertil. Steril., 72, 509–512.
7) Nakagawa, K., Yamano, S., Moride, N., Yamashita, M., Yoshizawa, M. and Aono, T. (2001): Effect of activation with Ca ionophore A23187 and puromycin on the development of human oocytes that failed to fertilize after intracytoplasmic sperm injection. Fertil. Steril., 76, 148–152.
8) Lu, Q., Zhao, Y., Gao, X., Li, Y., Ma, S., Mullen, S., Critser, J. K. and Chen, Z.-J. (2006): Combination of calcium ionophore A23187 with puromycin salvages human unfertilized oocytes after ICSI. Eur. J. Obstet. Gynecol. Reprod. Biol., 126, 72–76.
9) Lu, Q., Chen, X., Li, Y., Zhang, X.-H., Liang, R., Zhao, Y.-P., Wei, L.-H. and Shen, H. (2012): A live birth of activated one-day-old unfertilized oocyte for a patient who experienced repeatedly near-total fertilization failure after intracytoplasmic sperm injection. Chin. Med. J., 125, 546–548.
10) Economou, K. A., Christopikou, D., Tsorva, E., Davies, S., Mastrominas, M., Cazlaris, H., Koutsilieris, M., Angelogianni, P. and Loutradis, D. (2017). The combination of calcium ionophore A23187 and GM-CSF can safely salvage aged human unfertilized oocytes after ICSI. J. Assist. Reprod. Genet., 34, 33–41.
11) Shibahara, T., Fukasaku, Y., Hayashi, N., Miyazaki, N., Kawato, H. and Minoura, H. (2021): Early rescue oocyte activation for activation-impaired oocytes with no second polar body extrusion after intracytoplasmic sperm injection. J. Assist. Reprod. Genet., 38: 1061–1068.
12) Payne, D., Flaherty, S.P., Barry, M.F. and Matthews, C.D. (1997): Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. Hum. Reprod., 12, 532–541.
13) Liu, Y., Chapple, V., Roberts, P., Ali, J. and Matson, P. (2014): Time-lapse videography of human oocytes following intracytoplasmic sperm injection: Events up to the first cleavage division. Reprod. Biol., 14, 249–256.
14) Itoi, F., Asano, Y., Shimizu, M., Nagai, R., Saitou, K., Honnma, H. and Murata, Y. (2017): Clinical outcomes after IVF or ICSI using human blastocysts derived from oocytes containing aggregates of smooth endoplasmic reticulum. Reprod. Biomed. Online, 34, 337–344.
15) Huang, C.Y., Chen, G.Y., Shieh, M.L. and Li, H.Y. (2018): An extremely patient-friendly and efficient stimulation protocol for assisted reproductive technology in normal and high responders. Reprod. Biol. Endocrinol., 16, 1–7.
16) Veeck, L.L. (1999): An atlas of human gametes and conceptuses: an illustrated reference for assisted reproductive technology. Taylor & Francis, London.
17) Schoolcraft, W.B., Gardner, D.K., Lane, M., Schlenker, T., Hamilton, F. and Meldrum, D.R. (1999): Blastocyst culture and transfer: analysis of results and parameters affecting outcome in two in vitro fertilization programs. Fertil. Steril., 72, 604–609.
18) Kanda, Y. (2013): Investigation of the freely available easy-to-use software "EZR" for medical statistics. Bone Marrow Transplant., 48, 452–458.
19) Vanden Meerschaut, F., Nikiforaki, D., Heindryckx, B. and De Sutter, P. (2014): Assisted oocyte activation following ICSI fertilization failure. Reprod. Biomed. Online, 28, 560–571.
20) Miyazaki, S. and Ito, M. (2006): Calcium signals for egg activation in mammals. J. Pharmacol. Sci., 100, 545–552.
21) Drakopoulos, P., Blockeel, C., Stoop, D., Camus, M., De Vos, M., Tournaye, H. and Polyzos, N.P. (2016): Conventional ovarian stimulation and single embryo transfer for IVF/ICSI. How many oocytes do we need to maximize cumulative live birth rates after utilization of all fresh and frozen embryos? Hum. Reprod., 31, 370–376.
22) Law, Y.J., Zhang, N., Venetis, C.A., Chambers, G.M. and Harris, K. (2019): The number of oocytes associated with maximum cumulative live birth rates per aspiration depends on female age: A population study of 221 221 treatment cycles. Hum. Reprod., 34, 1778–1787.