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RESEARCH ARTICLE

Fertilization with human sperm bound to zona pellucida by pressing onto the oocyte membrane

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Abstract

This study aimed to determine whether fertilization can be obtained by assisted fusion of oocyte and sperm without breaking the oocyte membrane. A total of 79 infertile couples, each with at least one unfertilized oocyte after in vitro fertilization (IVF), were recruited. Sperm collected from the zona pellucida (ZP) were pressed onto the membrane of unfertilized oocytes at either 6 h or 24 h after IVF, a procedure that we designated as assisted sperm fusion insemination (ASFI). The results of ASFI were compared with those obtained in a previous trial on oocytes in which rescue intracytoplasmic sperm injection (ICSI) was performed at 6 h after IVF. Acrosome reaction (AR) rate of sperm bound to ZP, fertilization rate, degeneration rate, and blastocyst formation rate were evaluated. The AR rate of sperm collected from the ZP was significantly higher than that of the motile sperm recovered from around the oocytes but not bound to the ZP after IVF (98.0% vs. 28.6%). ASFI which was performed at 6 h after IVF yielded a mean fertilization rate of 73.4% (58/79), a degeneration rate of 0% (0/79) and a blastocyst formation rate of 60.8% (31/51). Rescue ICSI which was performed at 6 h after IVF yielded a mean fertilization rate of 70.0% (70/100), a degeneration rate of 4% (4/100) and a blastocyst formation rate of 42.4% (25/59). Binding of sperm to the ZP typically results in AR. ASFI with acrosome-reacted sperm collected from the ZP yielded the fertilization rates similar to those obtained with rescue ICSI.

Keywords Acrosome reaction · Zona pellucida · Rescue intracytoplasmic sperm injection · Oocyte membrane · Fertilization

Introduction

Total fertilization failure (TFF) after in vitro fertilization (IVF) is disappointing for infertile patients. Although TFF cycles have been reduced by introduction of intracytoplasmic sperm injection (ICSI), the complete elimination of TFF remains a challenge. Rescue ICSI, which is ICSI of unfertilized 1-day-old oocytes after conventional IVF, had been reported to solve TFF problems [1]. However, recent studies showed that the outcome of rescue ICSI is unsatisfactory, resulting in a poor fertilization rate [2–4] and a high degeneration rate [5]. To solve this problem, the method that rescue ICSI is performed at 6 h after insemination has been reported and this method improved the fertilization rate without raising three pronuclei rate [6, 7]. A micromanipulation technique that permitted fertilization without breaking of the oocyte membrane would be expected to reduce further the degeneration rate after ICSI. Subzonal insemination (SUZI) has been used to improve the treatment of male infertility [8]. SUZI is a fertilization method that does not break the oocyte membrane, thereby largely eliminating the possibility of oocyte degeneration; however, it had been reported that the fertilization rate with SUZI is lower than that with ICSI [9]. One of the reasons for the low fertilization rate after SUZI may be that the sperm swim freely in the perivitelline space. Therefore, we have attempted to fertilize the oocyte by artificially pressing sperm onto the oocyte membrane. Although mouse oocytes could be fertilized in the same technique, it has not been reported that human oocyte was fertilized in this method [10]. Furthermore, we have attempted to improve the fertilization rate using zona pellucida (ZP)-bound sperm that were collected from the ZP with an injection needle. The aim of this study was to

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investigate whether it is possible to employ ZP-bound sperm to fertilize the unfertilized oocyte at 6 h or 24 h after conventional IVF. Specifically, fertilization was attempted using the injection needle to collect ZP-bound sperm and subsequently press these sperm onto the oocyte membrane. We have designated this micromanipulation procedure: "assisted sperm fusion insemination" (ASFI).

Materials and methods

Between January 2019 and January 2020, a total of 115 oocytes from 79 patients were included in this study. We performed ASFI on unfertilized oocytes at 6 h after conventional IVF in 54 cycles (ASFI-6h). In addition, because of oocyte immaturity on the day of oocyte retrieval, we also performed ASFI at 24 h after conventional IVF in 27 cycles (ASFI-24h). The results of ASFI were compared with those obtained in a previous trial on oocytes from a different group of patients in which rescue ICSI was performed at 6 h after conventional IVF (ICSI-6h) and ICSI was performed at 24 h after oocyte retrieval because of immaturity of the oocyte on the day of oocyte retrieval (ICSI-24h). In ICSI-6h and ICSI-24h groups, a total of 174 oocytes from 106 patients were included between March 2012 and July 2019. This study was approved by the Ethics Committee at the Yanaihara Women's Clinic (YW18-02) and Gunma Paz University (PAZ18-22). Informed consent was obtained from all patients.

Sperm preparation

Semen samples were obtained by masturbation after 3–5 days of ejaculatory abstinence. For all samples, the sperm concentration and motility were assessed based on the World Health Organization criteria [11]. Semen was processed by density grade separation using Isolate® (Irvine Scientific, CA, USA). After centrifugation at 300*g* for 20 min, the supernatant was removed and 0.2 mL of HTF Medium (Irvine Scientific, CA, USA) with 10% serum substitute supplement (SSS) (Irvine Scientific, CA, USA) was pipetted over the pellet to facilitate swimming-up of the sperm. The sample then was incubated for 20 min at 37 °C, at which point 0.1 mL of the upper layer of the media was carefully collected.

Ovarian stimulation and insemination

Ovarian stimulation was performed with either a mild stimulation protocol using a GnRH antagonist or with a short agonist protocol. The mild stimulation was performed using 100 mg clomiphene citrate (Shionogi, Osaka, Japan) per day for 5 days between days 3 and 7 of the menstrual cycle and 150 IU of human menopausal gonadotropin on days 3, 5, and 7 of the menstrual cycle. Follicular development was followed by ultrasonography. When the size of the dominant follicles reached ≥ 14 mm in diameter, 0.25 mg of GnRH antagonist was injected daily until the day of a maturation trigger. When the size of the dominant follicles reached > 17 mm in diameter, 10,000 IU of human chronic gonadotropin (hCG) was administered, and oocyte retrieval was performed 35 h after hCG administration. Conventional IVF was performed within 3–4 h after oocyte retrieval. In detail, 160,000 motile sperm were suspended in 800 µL of Universal IVF Medium® (CooperSurgical, CT, USA) that were equilibrated in Nunc™ 4-Well Dishes for IVF (Thermo Fisher Scientific, MA, USA). One to three cumulus–oocyte complexes were put in these wells and incubated at 37 °C, 6% CO_2.

Collecting sperm bound to ZP and assessment of acrosomal status

Forty-nine motile sperm bound to the ZP from 17 patients were used for the assessment of acrosomal status. The acrosomal status of sperm was determined with fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) [12], obtained as Lectin from *Arachis hypogaea* (peanut), FITC conjugate, lyophilized powder (MilliporeSigma, MA, USA). The FITC-PNA, was dissolved to a final concentration of 40 µg/mL in phosphate-buffered saline (PBS) (−) containing 0.5 wt% bovine serum albumin. Propidium iodide (PI), obtained as cellstain PI solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), was used to stain the sperm nuclei. This solution was diluted in PBS (–) to yield PI at a final concentration of 0.05 µg/mL. Both motile sperm that were located around the oocytes but not bound to the ZP (control group) and motile sperm that were bound to the ZP (ZP group) were used for assessment of the acrosome reaction (AR). After a fertilization check, unfertilized oocytes were stabilized using a holding pipette (Kitazato Corporation, Shizuoka, Japan) and sperm bound to the ZP were kept away from the holding pipette. For the ZP group, the motile sperm bound to the ZP were aspirated directly using an injection pipette (CooperSurgical, CT, USA). Following sperm collection, mineral oil around the injection needle was removed by Multipurpose Handling Medium® (MHM) (Irvine Scientific, CA, USA) with SSS; the sperm then were dispensed in a 1-µL droplet of MHM on a MAS-GP type A-coated glass slide (Matsunami Glass, Osaka, Japan). To avoid sperm loss, the sperm were adhered to the slide by touching each sperm tail with the injection needle under the microscope. For the control group, motile sperm recovered from around the oocytes but not bound to the ZP also were collected using an injection needle and adhered on the slide as described above. The slides with adherent sperm were air-dried, fixed with 4% formaldehyde in PBS (−) for 15 min at

room temperature, washed with distilled water, dried at room temperature, incubated for 30 min with 15 µL of FITC-PNA at 37 °C in a moisture chamber, washed again with distilled water, and covered first with PI solution and then with a cover glass. The acrosomal status of the sperm was evaluated using a Nikon Eclipse Ci-s (Nikon Corporation, Tokyo, Japan) and a Nikon Intensilight Epi-fluorescence Illuminator C-HGF (Nikon Corporation, Tokyo, Japan) at a × 1000 magnification using an oil-covered objective.

ASFI procedure

We attempted to fertilize the oocytes that were defined as unfertilized oocytes after IVF using the following method. Three hours after conventional insemination, all inseminated oocytes were denuded of cumulus cells by glass pipettes and the presence of the second polar body was confirmed by microscopic inspection. Oocytes lacking the second polar body were incubated for another 3 h and the absence of the second polar body was reconfirmed. Oocytes exhibiting only one polar body were defined as unfertilized oocytes (MIIs) and used for ASFI (ASFI-6h). The immature oocytes in which the presence of the first polar body was not confirmed at 6 h after insemination were re-inseminated with 160,000 motile sperm for 17–18 h. Oocytes were examined again for evidence of fertilization at 23–24 h after the conventional IVF. When the immature oocytes matured into MIIs, we performed ASFI (ASFI-24h). After the fertilization check, an MII was stabilized using a holding pipette. A motile sperm bound to the ZP of the target oocyte was aspirated, tail first, into the tip of the injection needle without impeding sperm motility. The injection needle was pushed across the ZP into the perivitelline space and the tip of the injection needle was pressed against the oocyte membrane (Fig. 1a, b) until the curve of the tip of the injection needle matched that of the surface of the oocyte membrane. The sperm head was forced to adhere to the oocyte membrane for 30 s by applying pressure via the microinjector. The injection needle then was

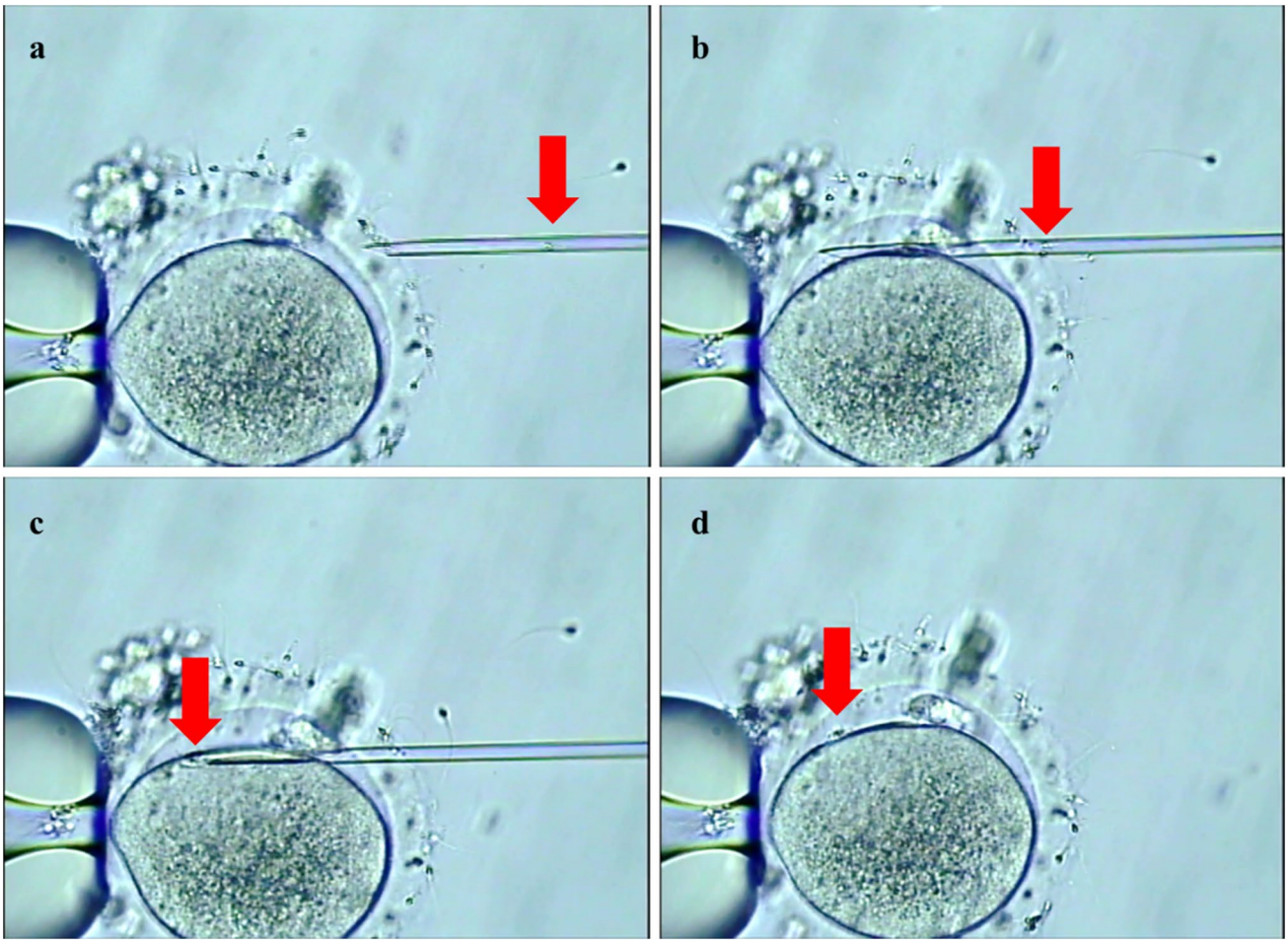

Fig. 1 Pressing the sperm onto the oocyte membrane (representative images). **a**, **b** Pushing the injection needle into the perivitelline space by pressing the tip of the injection needle against the oocyte membrane. **c** Pressing the sperm head onto the oocyte membrane for 30 s. **d** Adhesion of the sperm onto the oocyte membrane. Red arrows: the sperm collected from the zona pellucida

withdrawn gently (Fig. 1d). After withdrawal the injection needle, adherence of the sperm to the oocyte membrane was assessed. Sperm that did not swim despite continuing tail movement were considered to have adhered to the oocyte membrane.

Assessment of fertilization and embryonic development

Oocytes were cultured with Global® total® (CooperSurgical, CT, USA) and observed 16–19 h after completion of the ASFI procedure; survival, and the number of pronuclei (PN) were assessed with an EmbryoScope+ time-lapse system (Vitrolife, Göteborg, Sweden). The scoring criteria for cleavage-stage embryos suggested by Veeck [13] were used; embryos that scored as more than seven cells grade 2 on Day 3 were defined as morphologically good embryos. A subset of these embryos was frozen on Day 3 in case that the patient wanted; the remaining embryos were cultured until they reached the blastocyst stage and assessed for blastocyst formation rate. The scoring criteria for blastocysts suggested by Gardner [14] were used; embryos that scored as more than full blastocyst except CC were frozen on Day 5 or 6.

Statistical analysis

Mean values were compared using a Welch's *t* test; proportions were compared using a Fisher's exact test. Analyses were performed as two-tailed tests. A P value of < 0.05 was considered statistically significant. We conducted all analyses using R software, version 3.5.1 [15].

Results

Acrosomal status

Table 1 and Fig. 2 show the acrosomal status, as determined by FITC-PNA staining, of the ZP-bound vs. control sperm. The sperm nuclei were labeled with red fluorescence and the intact acrosomes were labeled with green fluorescence. Notably, the acrosomal region of the acrosome-reacted sperm did not exhibit labeling with green fluorescence. The AR rate of the ZP group was 98%, a value that was significantly ($P < 0.01$) greater than that of the control group (28.6%).

Table 1 Acrosome reaction of bound sperm in the ZP and control groups

	ZP group	Control group
No. of patients	17	
No. of motile sperm assessed	49	56
No. of acrosome-reacted sperm, *n* (%)	48 (98.0)[a]	16 (28.6)

ZP zona pellucida

[a] $P < 0.01$ compared to the control group

Fertilization and embryonic development

After pressing sperm onto the oocyte membrane, the sperm remained there for approximately 30 min, and then were incorporated into the oocyte membrane (Fig. 3). All sperm collected from the ZP adhered to the oocyte membrane after ASFI. The results of ASFI and ICSI are shown in Table 2. A total of 79 unfertilized MII oocytes were obtained from the 54 patients and subjected to ASFI-6h. The fertilization rate of ASFI-6h (73.4%) and ICSI-6h (70.0%) oocytes was significantly ($P < 0.05$) higher than that of ASFI-24h (41.7%) and ICSI-24h (47.3%) oocytes. The degeneration rate of ASFI-6h (0%), ICSI-6h (4%) and ASFI-24h (0%) oocytes was significantly ($P < 0.05$) lower than that of ICSI-24h (18.9%) oocytes.

Although there were no significant differences in one-pronucleus rate, three-pronuclei rate, and percentage of morphologically good embryos on Day 3 between any groups, the blastocyst formation rate was significantly ($P < 0.01$) higher in ASFI-6h (60.8%) and ICSI-6h (42.4%) compared to that in ICSI-24h (8.6%) (Table 2).

Discussion

In this study, we attempted fertilization of human oocytes by pressing, onto the oocyte membrane, sperm that had bound to the ZP. We found that the AR rate of the sperm bound to the ZP was higher than that reported previously [16–18]. Moreover, we succeeded in fertilizing the oocytes by pressing, onto the oocyte membrane, sperm that had bound to the ZP. The establishment of a fertilization method that uses a single sperm without breaking the oocyte membrane is important in infertility treatment because there is possibility of oocyte degeneration after ICSI.

The AR is a reaction that results in an exocytotic event and is required for fusion of the sperm and oocyte. Previous reports [16–18] have indicated AR rates of 22–48% for sperm bound to the ZP and subsequently collected by aspiration of the oocyte, but the present work yielded an AR rate of 98%. We hypothesize that a method employing aspiration of the oocyte is not appropriate for collection of sperm bound to the ZP. In contrast, there were two differences in the sperm-collecting method describe in our study. First, we directly aspirated the sperm bound to the ZP using the injection pipette. This method permits collection of the ZP-bound sperm only following confirmation of adhesion of the sperm head to the ZP. Second, we adhered the sperm to the

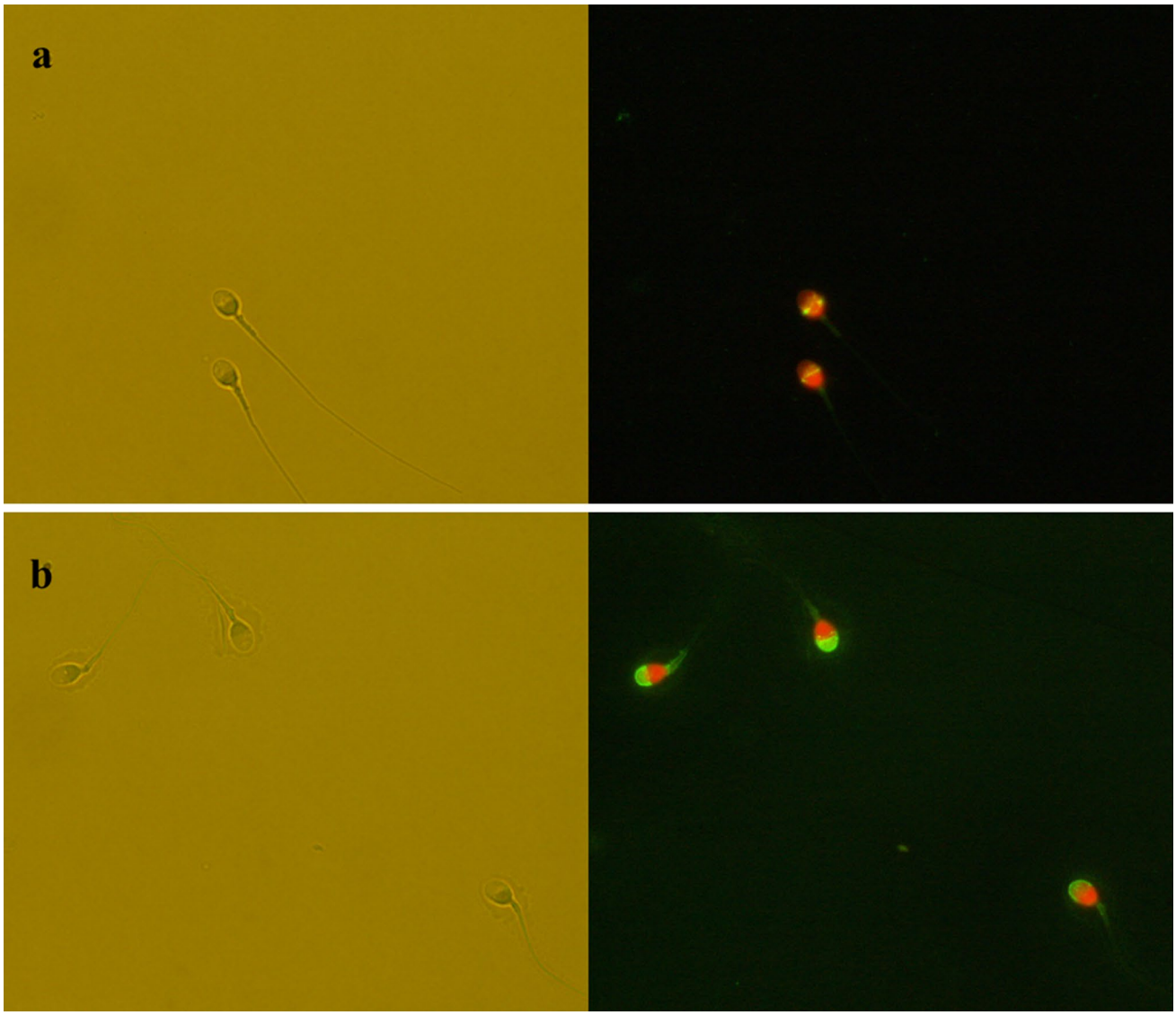

Fig. 2 Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) labeling (representative images). Acrosomal status of sperm bound and not bound to the zona pellucida (ZP) 24 h after insemination. Nuclei were labeled with propidium iodide (red fluorescence; acrosomes were labeled with FITC (green fluorescence). **a** Sperm collected from the ZP, bright field (left); two acrosome-reacted sperm are seen (right). **b** Sperm not bound to the ZP, bright field (left); three acrosome intact sperm are seen (right). Sperm with no green fluorescence over the acrosomal region were classified as reacted

slide by touching the sperm tail with the injection needle, a step performed under the microscope, to avoid sperm loss. This technique may not affect the result of the AR because the sperm in the control group also were treated with the same technique.

ASFI with the acrosome-reacted sperm that were collected from the ZP has some advantages. First, there is little risk of oocyte degeneration because this fertilization method permits fertilization of the oocyte without breaking the oocyte membrane. ASFI reduced the degeneration rate of the oocytes at 24 h after oocyte retrieval in this study. Second, given that ASFI is performed by pressing the acrosome-reacted sperm to the oocyte membrane, there is no possibility that any non-physiological substances, polyvinylpyrrolidone, or acrosomal enzymes are injected into the cytoplasm of the oocyte. It is inevitable that these substances are injected (with the sperm) into the cytoplasm of the oocyte in the course of ICSI. Because the effects of these substances on the next generation are not clear [19], injection of such substances into the cytoplasm of the oocyte should be avoided. Third, the oocyte can be fertilized with acrosome-reacted sperm. In ASFI, only acrosome-reacted sperm is permitted to combine with the oocyte, whereas in ICSI the acrosomal status of the sperm injected into the

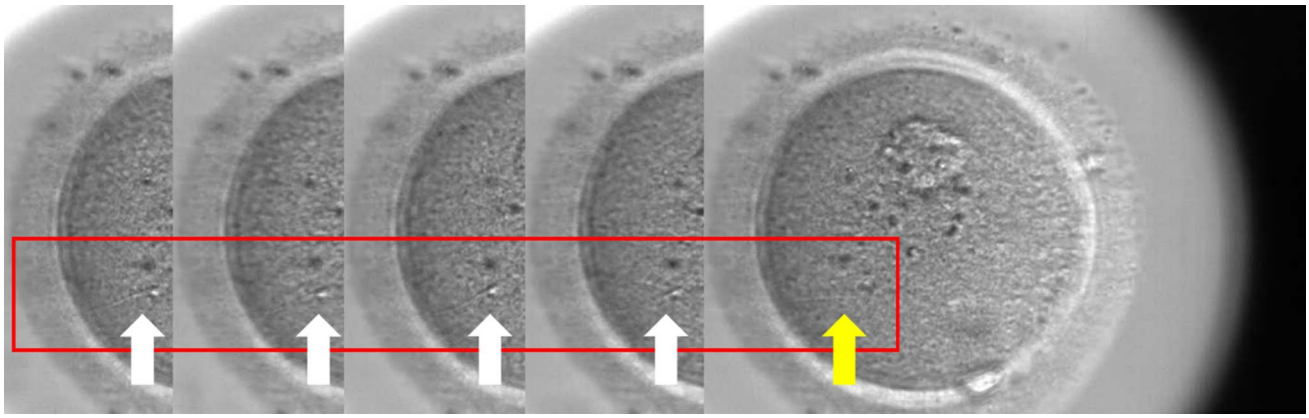

Fig. 3 A sperm that was adhered onto the oocyte membrane after ASFI (representative time series). The sperm remained in place for 30 min before being incorporated into the oocyte membrane. The red square shows the sperm state. White arrows: the sperm head and tail. Yellow arrow: Only the sperm tail remains visible after the fusion of the sperm head and the oocyte

Table 2 Results of ASFI and ICSI

	ASFI-6h	ICSI-6h	ASFI-24h	ICSI-24h
No. of cycles, *n*	54	53	27	55
Female age, years	36.1 ± 3.8[a]	37.7 ± 4.3	35.7 ± 4.7[a]	39.3 ± 4.3[b]
Male age, years	37.3 ± 4.7[c]	40.2 ± 5.6[d]	36.6 ± 5.8[c]	40.1 ± 4.6[d]
Semen volume, mL	2.5 ± 0.9	2.7 ± 1.3	2.7 ± 1.2	2.4 ± 1.1
Sperm density, 10^6/mL	81.3 ± 68.9[c]	88.8 ± 82.3	128.1 ± 72.1[a,d]	65.7 ± 55.1[b]
Sperm motility, %	55.2 ± 13.1	55.1 ± 17.3	53.8 ± 15.7	46.6 ± 20.1
No. of oocytes undergoing ASFI or ICSI	79	100	36	74
No. of adhered sperm	79 (100.0)	–	36 (100.0)	–
No. of fertilized oocytes with two pronuclei	58 (73.4)[c]	70 (70.0)[c]	15 (41.7)[d]	35 (47.3)[d]
No. of fertilized oocytes with one pronucleus	4 (5.1)	6 (6.0)	4 (11.1)	8 (10.8)
No. of fertilized oocytes with three pronuclei	4 (5.1)	14 (14.0)	3 (8.3)	6 (8.1)
No. of degenerated oocytes	0 (0)[c]	4 (4.0)[c]	0 (0)[c]	14 (18.9)[d]
No. of morphologically good embryos on Day 3	36 (62.1)	36 (51.4)	–	–
No. of blastocysts	31 (60.8)[a,c]	25 (42.4)[a]	2 (13.3)[d]	3 (8.6)[b]

Data are presented as mean ± standard deviation or *n* (%). ASFI was performed on unfertilized oocytes from these patients at 6 h or 24 h after conventional IVF. ICSI-6h was performed on unfertilized oocytes from these patients at 6 h after conventional IVF. ICSI-24h was performed at 24 h after oocyte retrieval

[a,b]Values with different superscripts in the same row are significantly different ($P < 0.01$)

[c,d]Values with different superscripts in the same row are significantly different ($P < 0.05$)

oocyte is unknown. Although staining of the sperm would allow clarification of the acrosomal status, the stained sperm then cannot be used for the actual treatment. However, ICSI using ZP-bound sperm has been shown to yield higher developmental capacity [20] and higher implantation and clinical pregnancy rates [21, 22] compared to ICSI using conventionally selected sperm. These reports suggest that the use of acrosome-reacted sperm may provide improved clinical results.

Some limitations exist in the present study. First, given that the sperm need to combine with the ZP, this method may not be appropriate for male infertility patients with severe oligospermia. A method of artificially inducing AR would need to be established to solve this problem. Although progesterone [23, 24], A23187, and culture media [25] induce AR, the resulting AR rates are insufficient. Therefore, a new method that induces high rates of AR would be needed. Otherwise, a method for assessing the acrosomal status of the sperm without staining is required. Second, some oocytes remained unfertilized even after adhesion of the sperm to the oocyte membrane. Notably, failure to fertilize was seen with sperm bound at some depths within the ZP, that is, sperm for which the sperm head absolutely penetrated the ZP, as opposed to those in which half of the sperm head or

only the tip of the sperm head adhered to the ZP (data not shown). Although all of these sperm were able to adhere to the oocyte membrane, fertilization appeared to depend on the sperm head invasion depth to the ZP. Further studies will be needed to clarify this issue.

In conclusion, we succeeded in fertilizing oocytes by pressing the sperm collected from the ZP against the oocyte membrane without breaking the membrane itself. This new method, which we designate ASFI, is expected to improve the survival rate of the oocyte and to increase the number of the embryos that are available for implantation. In addition, ASFI may contribute to the elucidation of the mechanism of fertilization, given that the technique permits observation of the process of fusion between the oocyte and sperm.

Compliance with ethical standards

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

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the national committee, and were approved by the Ethics Committee at the Yanaihara Women's Clinic (YW18-02) and Gunma Paz University (PAZ18-22).

Informed consent Informed consent was obtained from all individual participants included in the study.

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