



Clinical Outcomes of Rescue Intracytoplasmic Sperm Injection at Different Timings Following In Vitro Fertilization

Yuki Shiraiwa¹, Noritoshi Enatsu^{1*}, Kazuki Yamagami¹, Koyu Furuhashi¹, Toshiroh Iwasaki¹, Junko Otsuki^{1,2}, Masahide Shiotani¹

1- Hanabusa Women's Clinic, Hyogo, Japan

2- Okayama University Assisted Reproduction Technology Center, Okayama, Japan

Abstract

Background: Although rescue intracytoplasmic sperm injection (r-ICSI) is extensively used worldwide, the indication of r-ICSI and its optimal timing remains obscure. This study aimed to assess the outcomes of r-ICSI following *in vitro* fertilization in different timings when fertilization is confirmed.

Methods: This study included 5,156 cycles (47,785 eggs). Fertilization was confirmed by polar body analysis after 4 and 6 *hr* of coincubation of the sperm and oocyte. Oocytes that underwent IVF were divided into two groups based on the time when a second polar body was detected in more than 30% of all oocytes (Four-*hr* group and six-*hr* group). If the second polar body was not detected or was present in less than 30% of all oocytes after six *hr* of coincubation, rescue-ICSI (r-ICSI) was performed for oocytes without a second polar body (r-ICSI group).

Results: The fertilization rates of two pronuclear (2PN) oocytes in the three groups (Four-*hr* group, six-*hr* group, and r-ICSI group) were 70.7%, 51.3%, and 58.0%, respectively. The blastocyst formation rates were 62.8%, 53.4%, and 42.9%, respectively.

Conclusion: Performing r-ICSI after six *hr* of coincubation can salvage cases with fertilization failure in IVF. The higher fertilization rate of r-ICSI indicates that all oocytes without signs of fertilization after six *hr* of coincubation should undergo r-ICSI.

Keywords: Assisted reproductive techniques, Fertilization failure, In vitro fertilization, Infertility, Intracytoplasmic sperm injection.

To cite this article: Shiraiwa Y, Enatsu N, Yamagami K, Furuhashi K, Iwasaki T, Otsuki J, et al. Clinical Outcomes of Rescue Intracytoplasmic Sperm Injection at Different Timings Following In Vitro Fertilization. *J Reprod Infertil.* 2021;22(4):251-257. <http://dx.doi.org/10.18502/jri.v22i4.7650>.

Introduction

Intracytoplasmic sperm injection (ICSI) was developed as a means to help couples with male factor infertility, such as oligozoospermia, asthenozoospermia, and azoospermia. Many studies have reported that in patients with severe male factor infertility, the ICSI procedure results in a higher fertilization rate than conventional *in vitro* fertilization (IVF). Therefore, the ICSI technique has been universally promoted, and its use is increasing worldwide.

ICSI is indicated not only for patients with severe male factor but also for cases with fertilization failure. Unexpected fertilization failure sometimes occurs in conventional IVF cycles. The incidence of total fertilization failure has been reported to range from 4%-16% (1, 2). To salvage fertilization failure, performing ICSI for unfertilized 1-day-old oocytes, called rescue-ICSI (r-ICSI), has been frequently suggested (3, 4). However, according to previous studies, the outcomes

* Corresponding Author:
Noritoshi Enatsu, Hanabusa
Women's Clinic, Hyogo,
Japan
E-mail:
enatsunoritoshi@
hanabusaclinic.com

Received: Oct. 20, 2020

Accepted: Mar. 15, 2021

of r-ICSI were unsatisfactory and resulted in a poor clinical pregnancy rate (5, 6). These outcomes may be caused by the *in vitro* aging of cultured oocytes before ICSI, which could cause increased cytogenetic abnormalities and a lower fertilization rate. For such reasons, r-ICSI was performed earlier on the same day as oocyte pick up (OPU) and showed superior outcomes compared with 1-day-old r-ICSI (7-9).

However, there are some concerns regarding early r-ICSI. For example, performing r-ICSI on oocytes that are already in the process of fertilization could lead to polyspermy, which in most cases results in the formation of three- pronuclear (3PN) zygotes. It has been reported that 22% of oocytes release their second polar body within 2–4 *hr* and 66% release it at four *hr* (10). Therefore, early r-ICSI can overlook the possible delay in the release of the second polar body that occurs in up to 10% of oocytes. A similar concern is the fragmentation of the first polar body in some oocytes, which makes it difficult to distinguish the second polar body when r-ICSI is attempted (5). R-ICSI on these oocytes will lead to the formation of 3PN zygotes. In summary, the indication of r-ICSI at a later timing may lead to oocyte aging. Conversely, the indication of r-ICSI at an earlier timing may lead to 3PN zygotes. Therefore, the optimal timing for r-ICSI remains obscure. In this study, an attempt was made to retrospectively analyze the outcome of r-ICSI to identify an optimal strategy in performing r-ICSI.

Methods

This retrospective study included 5,156 consecutive patients with OPU cycles (47,785 eggs) who underwent IVF and consequential r-ICSI procedures in Hanabusa Women's Clinic between June 2012 and December 2017. All the patients who underwent IVF with at least four metaphase II (MII) oocytes were available for r-ICSI in case fertilization failure was confirmed. The criteria used in the Clinic for performing r-ICSI were based on the two-step observation of fertility signs (The presence of two or more polar bodies) 4 and 6 *hr* after sperm–oocyte coincubation. To avoid duplicated insemination of sperm, r-ICSI was performed only in cases with a fertilization rate of 30% or less after 6 *hr* of sperm–oocyte coincubation. The observation duration and cutoff point of 30% were based on a previous report and the laboratory experience (10). The purpose of the current study was performing a retrospective analysis

of 5,156 IVF cycles and 225 subsequent r-ICSI cycles by observing polar bodies (PB) at different timings to determine the optimal timing and indications for r-ICSI. All the procedures in this study were not experimental and have already been performed in many IVF centers under different criteria. Data were collected and anonymized in accordance with the ethical standards of the ethics committee as per the principles originating from the 1964 Declaration of Helsinki and its later amendments. This study was approved by the ethics committee of Hanabusa Women's Clinic, which consists of members chosen from our institute and a third-party medical institute (Approval No. 2020-07).

After oocyte retrieval, the oocytes were washed in the medium (Universal IVF medium®, Origio, Japan) and pre-cultured for 2.5 *hr* in the CO₂ incubator (Origio, Japan) under CO₂, O₂, and N₂ concentrations of 6.0%–5.5%, 5%, and 89.9%–89%, respectively. Semen samples were collected in sterile containers by masturbation. After liquefaction at room temperature, the samples were assessed for semen analysis using a computer-assisted sperm analysis (SMAS®, DITECT, Japan). Gradient separation was used with a centrifuge for 15 *min* at 300×*g*. A final concentration of 5–20×10⁴ motile *sperm/ml* was used for insemination. Cumulus oocyte complexes were inseminated in four-well plates (Falcon, Japan) containing 1 *ml* of fertilization medium (Universal IVF medium) covered with oil (Light mineral oil, Irvine Scientific, Japan) in the CO₂ incubator (Origio, Japan) at an air temperature of 37°C. Four *hr* after insemination, the cumulus complex was removed, and fertilization was assessed with 300–400× magnification using an inverted microscope. As shown in figure 1, the embryos were divided into three groups according to the timeline-based strategy of r-ICSI according to the fertilization rate. Fertilization failure was identified in cases that lacked a PN and a second polar body in the perivitelline space. If the fertilization rate was over 30% among all MII oocytes in the individual cycle, r-ICSI was not performed, and sperm–oocyte coincubation continued (Four-*hr* group). If the fertilization rate was 30% or less four *hr* after insemination, additional insemination was carried out. After six *hr* of insemination (An additional two *hr*), fertilization was assessed again, and sperm–oocyte coincubation was continued when the fertilization rate was over 30% (Six-*hr* group). If the fertilization rate was 30% or less, r-ICSI

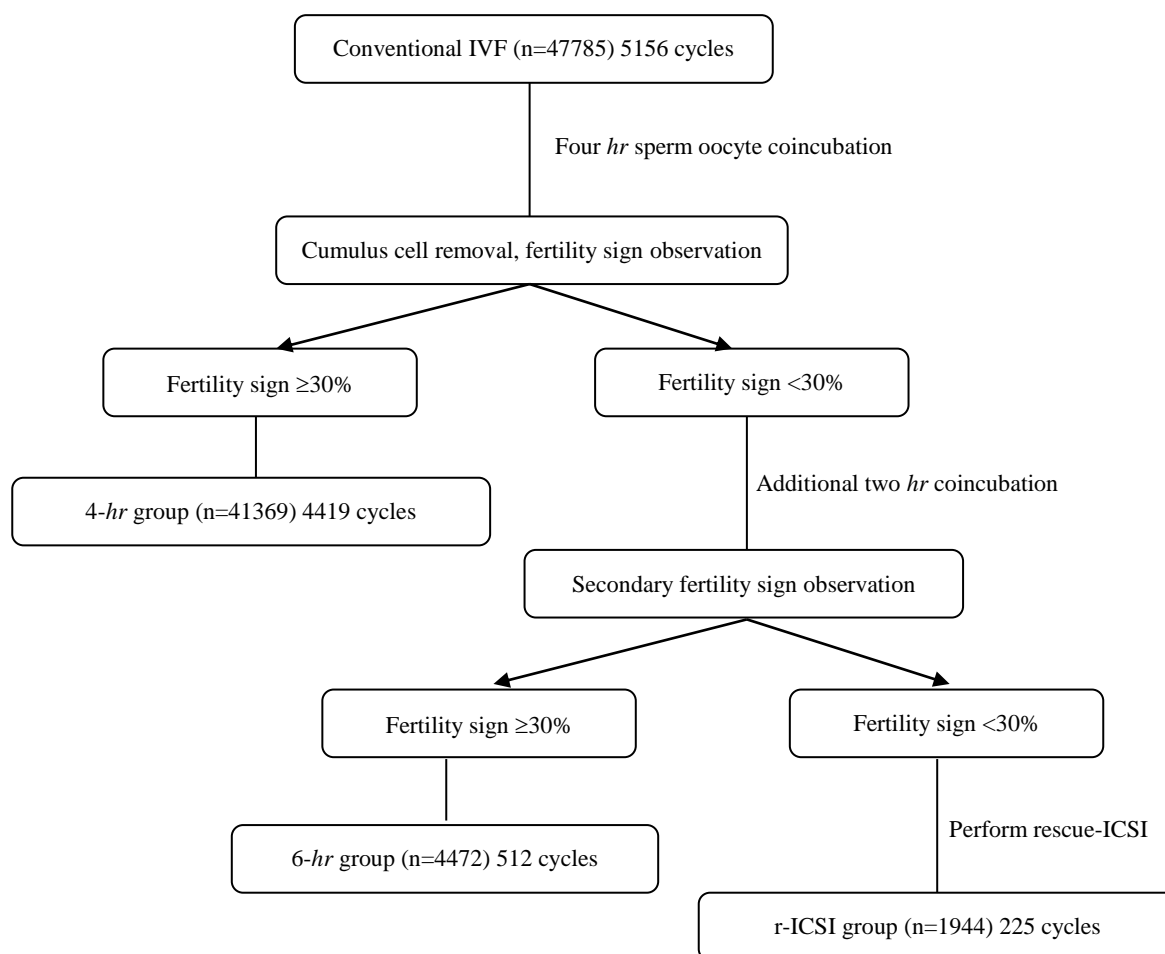


Figure 1. Timeline-based strategy of rescue-ICSI and grouping. The cycles were divided into three groups based on the two-step observation of fertilization signs (second polar body). To avoid duplicated sperm insemination, r-ICSI was only performed when the fertilization sign was detected in <30% of all oocytes after 6 hr of sperm oocyte co-incubation

was performed on those MII oocytes that did not show a second polar body (r-ICSI group). Fertilization was assessed 20 hr after insemination, and normal fertilization was defined as the detection of two individual PN formations. Embryos were examined at the cleavage stage (Day 2) and blastocyst stage (Day 5, 6) and transferred or cryopreserved according to the patients' requests after obtaining the informed consent. Embryos at the blastocyst stage were graded on the basis of Gardner's classification (11) and blastocysts with a grade of 3BB or better on day 5 were considered good quality blastocysts.

Statistical analysis was done using the student t-test, one way ANOVA and chi-square test, and $p < 0.001$ and $p < 0.01$ were considered statistically significant. All statistical analyses were performed with Microsoft Excel 2016 and EZR (Saitama Medical Center, Japan), a graphical user interface

for R (the R Foundation for Statistical Computing, Austria) (12).

Results

The results of timeline-based treatment strategy and the number of oocytes included in each group are summarized in figure 1. Of the 5,156 total cycles, 4,419 cycles (85.7%) were identified as being 2PB in more than 30% of the oocytes after 4 hr of co-incubation (4-hr group). Among the remaining 737 cycles, 512 displayed confirmed fertilization signs in more than 30% of the oocytes after 2 additional hours of observation (6-hr group). R-ICSI was performed in 225 (4.4%) of the total IVF cycles. Patient characteristics, including patients' age, number of assisted reproductive technology cycles, and number of oocytes retrieved did not differ among the three groups (Table 1).

Table 1. Patient characteristics

	Four- <i>hr</i> group	Six- <i>hr</i> group	r-ICSI group	p-value *
No. of patients	3386	475	214	-
No. of cycles	4419	512	225	-
Patient age (mean±SD) (years)	36.2±4.5	36.9±4.5	36.3±4.6	0.68
No. of ART history (mean±SD)	2.1±2.3	2.6±3.0	2.4±2.8	0.17
No. of oocytes retrieved (mean±SD)	9.6±5.4	9.0±4.8	8.9±4.7	0.09
No. of fresh embryo transfer cycles	682	79	35	
No. of frozen cycles	3551	380	159	

SD: Standard Deviation, ART: Assisted Reproductive Technology, r-ICSI: Rescue Intracytoplasmic Sperm Injection
* Calculated using analysis of variance

Table 2. Comparison of fertilization outcomes in the three groups

	Four- <i>hr</i> group	Six- <i>hr</i> group	r-ICSI group		
			IVF + r-ICSI	IVF	r-ICSI
No. of cycles	4419	512		225	
Oocytes underwent IVF	41369	4472	886	886	-
Oocytes underwent rescue-ICSI	-	-	1058	-	1058
Day 0					
Degenerated/immature oocytes (%)	8026 (19.4) ^a	930 (20.8)	489 (25.2)	489 (25.2)	-
OPN 1PB (%)	8652 (20.9) ^a	1366 (30.5) ^a	1058 (54.4)	-	1058 (54.4)
OPN 2PB (%)	22907 (55.4) ^a	1952 (43.6) ^a	226 (11.6)	226 (11.6)	-
OPN unclear PB (%)	489 (1.2) ^a	105 (2.3)	135 (6.9)	135 (6.9)	-
OPN >2PB (%)	1295 (3.1)	119 (2.7)	36 (1.9)	36 (1.9)	-
Day 1					
2PN (%) *	23560 (70.7) ^a	1817 (51.3) ^b	844 (58.0)	145 (16.7)	699 (66.1)
3PN (%) *	2749 (8.2) ^b	125 (4.7)	67 (4.6)	12 (1.4)	55 (5.2)
1PN (%) *	1995 (6.0)	330 (9.3)	137 (9.4)	45 (5.1)	92 (8.7)

PN: Pronucleus; PB: Polar Body; r-ICSI: Rescue Intracytoplasmic Injection; IVF: In vitro fertilization

* The 2PN, 3PN, and 1PN rates on day 1 were calculated on the basis of the number of oocytes without degenerated/immature oocytes as the denominator

a: p<0.001 by chi-square test for comparison with the r-ICSI group (IVF + r-ICSI)

b: p<0.01 by chi-square test for comparison with the r-ICSI group (IVF + r-ICSI)

Table 2 shows the laboratory outcomes in three groups. The day 0 results revealed that the 2PB rates were 55.4%, 43.6%, and 11.6%, and the 1PB rates were 20.9%, 30.5%, and 54.4% in the 4-*hr*, 6-*hr*, and r-ICSI groups, respectively. The day 1 results were calculated based on the number of oocytes without degeneration or the number of immature oocytes as the denominator. The normal fertilization (Two pronuclei [2PN]) rates on day 1 were 70.7%, 51.3%, and 58.0% in the 4-*hr*, 6-*hr*, and r-ICSI groups, respectively. In the r-ICSI group, the low fertilization rate of IVF (16.7%) was increased by r-ICSI (66.1%), resulting in an overall fertilization rate of 58.0%, which was higher than the 6-*hr* group.

Table 3 reveals the outcomes of the embryo culture according to the cleavage stage and blastocyst formation stage with different numbers of pronuclei on day 1 after insemination. Total blastocyst formation rates of 62.8%, 53.4%, and 42.9% were reported for the 4-*hr*, 6-*hr*, and r-ICSI groups, respectively. The blastocyst formation rate was the highest in normally fertilized (2PN) embryos in all groups (66.4%, 63.9%, and 52.0%, respectively). When compared with OPN embryos, the blastocyst formation rate was significantly higher in the 4-*hr* group (43.8%) than in the other groups (10.6% and 5.9%). Similarly, the blastocyst formation rate in 1PN embryos was higher in the 4-*hr* group than in the other groups.

Table 3. Outcomes of cleavage-stage embryos and blastocysts with different numbers of pronuclei at day one after insemination

	Four-hr group	Six-hr group	r-ICSI group		
			IVF + r-ICSI	IVF	r-ICSI
2PN					
No. of matured oocytes	24391	1877	846	147	699
No. of cleaved embryos (%)	23085 (94.6) ^a	1757 (93.6) ^b	763 (90.2)	128 (87.1)	635 (90.8)
No. of embryos continued developing during culture after cleavage	18435	1375	604	107	498
No. of blastocysts at day 5 (%)	10706 (58.1) ^a	730 (53.1) ^a	227 (37.6)	44 (41.1)	183 (36.7)
No. of blastocysts at day 6 (%)	1542 (8.4) ^b	149 (10.8) ^b	87 (14.4)	25 (23.4)	62 (12.4)
No. of total blastocysts (%)	12248 (66.4) ^a	879 (63.9) ^a	314 (52.0)	69 (65.1)	245 (49.2)
0PN					
No. of matured oocytes	6355	1400	477	331	146
No. of cleaved embryos (%)	1602 (25.2) ^a	142 (10.1) ^a	71 (14.9)	49 (14.8)	22 (15.1)
No. of embryos continued developing during culture after cleavage	1424	123	68	47	21
No. of blastocysts at day 5 (%)	546 (38.3) ^a	8 (6.5)	3 (4.4)	2 (4.3)	1 (4.8)
No. of blastocysts at day 6 (%)	78 (5.5)	5 (4.1)	1 (1.5)	0 (0)	1 (4.8)
No. of total blastocysts (%)	624 (43.8) ^a	13 (10.6) ^b	4 (5.9)	2 (4.3)	2 (9.5)
1PN					
No. of matured oocytes	2146	349	145	53	92
No. of cleaved embryos (%)	1244 (58.0) ^a	272 (77.9) ^b	107 (73.8)	42 (79.2)	65 (70.7)
No. of embryos continued developing during culture after cleavage	1169	256	105	41	64
No. of blastocysts at day 5 (%)	223 (19.1) ^b	23 (9.0)	9 (8.6)	5 (12.2)	4 (6.3)
No. of blastocysts at day 6 (%)	102 (8.7)	21 (8.2)	6 (5.7)	5 (12.2)	1 (1.6)
No. of total blastocysts (%)	325 (27.8) ^b	44 (17.2)	15 (14.3)	10 (24.4)	5 (7.8)
Total (2PN + 0PN + 1PN)					
No. of matured oocytes	32892	3626	1468	531	937
No. of cleaved embryos (%)	25931 (78.8) ^a	2171 (59.9) ^b	941 (64.1)	219 (41.2)	722 (77.1)
No. of embryos continued developing during culture after cleavage	21028	1754	777	194	583
No. of blastocysts at day 5 (%)	11475 (54.6) ^a	761 (43.4) ^b	239 (30.8)	51 (26.3)	188 (32.2)
No. of blastocysts at day 6 (%)	1722 (8.2)	175 (10.0)	94 (12.1)	30 (15.5)	64 (11.0)
No. of total blastocysts (%)	13797 (62.8) ^a	936 (53.4) ^a	333 (42.9)	81 (41.8)	252 (43.2)

r-ICSI: Rescue Intracytoplasmic Sperm Injection; IVF: In vitro Fertilization; PN: Pronucleus; PB: Polar Body

a: $p < 0.001$ by chi-square test for comparison with the r-ICSI group (IVF + r-ICSI)

b: $p < 0.01$ by chi-square test for comparison with the r-ICSI group (IVF + r-ICSI)

Discussion

ICSI has been confirmed to increase the likelihood of fertilization in the context of male factor infertility (13). However, there is no clear evidence of a benefit in using ICSI over conventional IVF with non-male factor infertility (14). Despite these facts, the popularity of ICSI has drastically increased worldwide, even in couples with non-male factor infertility. One of the main reasons why ICSI with non-male factor infertility has become so popular is unexpected fertilization failure. Therefore, proper handling in unexpected fer-

tilization failure will reduce the number of unnecessary cases of ICSI. Unexpected fertilization failure occurs in 4–16% of conventional IVF cycles (1, 2). Various methods, such as split-ICSI and 1-day-old ICSI, have been proposed to improve the oocyte fertilization rate after IVF. Among these methods, performing ICSI for 1-day-old unfertilized oocytes after IVF, called r-ICSI, has been frequently suggested. However, the *in vitro* aging of cultured oocytes before ICSI could cause a lower fertilization rate (4, 6). Therefore, to prevent the aging of oocytes, the early identification

of fertilization signs (two or more polar bodies) after 6 *hr* of insemination was introduced (5).

The most significant concern related to early r-ICSI is the occurrence of artificial 3PN zygotes by r-ICSI. The incidence of 3PN zygotes is known to have a negative impact on the outcomes of IVF and must be avoided as much as possible (15). Artificial 3PN zygotes occur when the fertilized oocytes are mistakenly observed as 1PB gametes when fertilization is confirmed due to the delayed release of the second PB. However, the 3PN ratio among r-ICSI was reported to be 6.6%, which is similar to that of the polyspermy rate in conventional IVF (Approximately 7%) (16). Similarly, the present study reported a low 3PN rate of 4.6%, which is even lower than those in the 4-*hr* (8.2%) and 6-*hr* (4.7%) groups. These results indicate that the incidence of artificial 3PN zygotes is not significant in r-ICSI after 6 *hr* of insemination.

Another area of interest is whether a shorter insemination of 4 *hr* is sufficient. To our knowledge, in no previous studies, r-ICSI was performed after less than 6 *hr* of incubation (17). Theoretically, 22% of zygotes release their second PB within 2–4 *hr*, and 66% release it at 4 *hr* (10). These facts indicate that the delayed release of the second PN could occur in up to 44% of cases, which could result in an artificial 3PN zygote. In the present study, the 2PN rate was 55.4% in four-*hr* group and 43.6% in the six-*hr* group when fertilization was confirmed, and the 2PN rates at day 1 were 70.7% and 51.3%, respectively. These results indicate that approximately 15% of oocytes were mistakenly observed as 1PN at 4 *hr* after insemination. Therefore, confirmation of fertilization signs at 4 *hr* is considered too early.

The last topic of interest is whether r-ICSI should be performed on all oocytes without fertilization at 6 *hr* after incubation. A recent study reported that r-ICSI is only recommended for cases with IVF fertilization rates <25%, considering the risk of artificial 3PN zygote in cases with IVF fertilization rates >25% (18). In the present study, in the comparison between the six-*hr* and r-ICSI groups, the 2PB rates at 6 *hr* after insemination were 43.6% and 11.6%, respectively, indicating a distinct fertilization disorder in the r-ICSI group. However, the 2PN rates at day 1 were 51.3% and 58.0%, respectively, reflecting reverse results. Such a higher fertilization rate in the r-ICSI group was apparently due to the performance of ICSI on unfertilized oocytes. In fact, the 2PN rate at day 1 was much higher in oocytes that underwent ICSI

(66.1%) than that of oocytes that underwent IVF (16.7%). Considering the fact that only 5.1% of oocytes were identified as 2PN on day 1 after being confirmed as unfertilized oocytes at 6 *hr*, delayed appearance of 2PB after 6 *hr* of incubation is rare. Therefore, performing r-ICSI for all unfertilized oocytes will be more beneficial than harmful, even if the 2PB rate was >30% at 6 *hr* after insemination.

It is important to emphasize that the current study had several limitations. This study included cases that underwent r-ICSI only when the fertilization rate was 30% or less after 6 *hr* of sperm–oocyte incubation. Furthermore, there are no data on the outcomes of r-ICSI at different timings, such as 8 *hr* or longer. However, considering the complicated logistic implications of evaluating oocytes at 8 or 10 *hr* after insemination with respect to the daily routine in IVF laboratories, it would be less cost effective to wait for longer than 6 *hr*.

Conclusion

In conclusion, r-ICSI is effective for salvaging unexpected fertilization failure in IVF. Confirmation of fertilization signs after 4 *hr* of sperm–oocyte incubation may misevaluate the fertilization sign in cases with late appearance of PB with normal fertilization, which could lead to 3PN zygote if r-ICSI was applied. Conversely, 1PB oocyte after 6 *hr* of sperm–oocyte incubation has little chance of becoming 2PN. Therefore, r-ICSI should be performed after 6 *hr* of incubation, and all oocytes without signs of fertilization at that time should undergo r-ICSI.

Acknowledgement

Shiraiwa and Furuhashi designed the project and collected data for the study. Enatsu and Yamagami contributed to data analysis and manuscript preparation. Iwasaki and Otsuki contributed to data analysis and editing of the manuscript. Shiotani was the study organizer and supervised this study.

The authors would like to thank Enago (www.enago.jp) for the English language review.

Conflict of Interest

The authors declare that they have no competing interests.

References

1. Barlow P, Englert Y, Puissant F, Lejeune B, Del-

- vigne A, Van Rysselberge M, et al. Fertilization failure in IVF: why and what next? *Hum Reprod.* 1990;5(4):451-6.
2. Molloy D, Harrison K, Breen T, Hennessey J. The predictive value of idiopathic failure to fertilize on the first in vitro fertilization attempt. *Fertil Steril.* 1991;56(2):285-9.
 3. Bell H, Garcia RB. ICSI of unfertilized oocytes after IVF insemination: are the traditional markers of fertilization adequate? *Hum Reprod.* 1995;10(3):491-3.
 4. Nagy ZP, Joris H, Liu J, Staessen C, Devroey P, Van Steirteghem AC. Intracytoplasmic single sperm injection of 1-day-old unfertilized human oocytes. *Hum Reprod.* 1993;8(12):2180-4.
 5. Chen C, Kattera S. Rescue ICSI of oocytes that failed to extrude the second polar body 6 h post-insemination in conventional IVF. *Hum Reprod.* 2003;18(10):2118-21.
 6. Kuczyński W, Dhont M, Grygoruk C, Pietrewicz P, Redzko S, Szamatowicz M. Rescue ICSI of unfertilized oocytes after IVF. *Hum Reprod.* 2002;17(9):2423-7.
 7. Zhu Lx, Ren Xi, Wu L, Hu J, Li Yf, Zhang Hw, et al. Rescue ICSI: Choose the Optimal Rescue Window before Oocyte Aging. *J Reprod Contraception.* 2011;22(1):29-36.
 8. Zhu L, Xi Q, Nie R, Chen W, Zhang H, Li Y. Rescue intracytoplasmic sperm injection: a prospective randomized study. *J Reprod Med.* 2011;56(9-10):410-4.
 9. Dai SJ, Qiao YH, Jin HX, Xin ZM, Su YC, Sun YP, et al. Effect of coincubation time of sperm-oocytes on fertilization, embryonic development, and subsequent pregnancy outcome. *Syst Biol Reprod Med.* 2012;58(6):348-53.
 10. Nagy ZP, Liu J, Joris H, Devroey P, Van Steirteghem A. Time-course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection. *Hum Reprod.* 1994;9(9):1743-8.
 11. Gardner DK, Schoolcraft WB. Culture and transfer of human blastocysts. *Curr Opin Obstet Gynecol.* 1999;11(3):307-11.
 12. Kanda Y. Investigation of the freely available easy-to-use software 'EZ' for medical statistics. *Bone Marrow Transplant.* 2013;48(3):452-8.
 13. Boulet SL, Mehta A, Kissin DM, Warner L, Kawwass JF, Jamieson DJ. Trends in use of and reproductive outcomes associated with intracytoplasmic sperm injection. *JAMA.* 2015;313(3):255-63.
 14. Li Z, Wang A, Bowman M, Hammarberg K, Farquhar C, Johnson L, et al. ICSI does not increase the cumulative live birth rate in non-male factor infertility. *Hum Reprod.* 2018;33(7):1322-30.
 15. Chen W, Bai H, Li M, Xue X, Shi J. Effects of three pro-nuclei (3PN) incidence on laboratory and clinical outcomes after early rescue intracytoplasmic sperm injection (rescue-ICSI): an analysis of a 5-year period. *Gynecol Endocrinol.* 2021;37(2):137-40.
 16. Xia P. Biology of polyspermy in IVF and its clinical indication. *Curr Obstet Gynecol Rep.* 2013;2(4):226-31.
 17. Beck-Fruchter R, Lavee M, Weiss A, Geslevich Y, Shalev E. Rescue intracytoplasmic sperm injection: a systematic review. *Fertil Steril.* 2014;101(3):690-8.
 18. Cao S, Wu X, Zhao C, Zhou L, Zhang J, Ling X. Determining the need for rescue intracytoplasmic sperm injection in partial fertilisation failure during a conventional IVF cycle. *Andrologia.* 2016;48(10):1138-44.