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ASSISTED REPRODUCTION TECHNOLOGIES

Failure of complete hatching of ICSI-derived human blastocyst by cell herniation via small slit and insufficient expansion despite ongoing cell proliferation

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Abstract

Purpose To assess the effect of intracytoplasmic sperm injection (ICSI) on embryo hatching and visualise the effects of zona thinning (ZT) on the embryo using time-lapse monitoring.

Methods In vitro fertilisation (IVF) (n = 178) and ICSI (n = 110)-derived cryopreserved blastocysts were donated by patients who previously had a baby. This study investigated the impacts of IVF, ICSI, laser-assisted hatching by ZT and formation of ICSI penetration trace on zona pellucida of IVF-derived blastocyst on blastcyst diameter, the estimated number of trophectoderm (TE) cells and completed hatching rate.

Results The completed hatching rate and diameters of the completely hatched blastocysts at hatching commencement and at the maximum expansion were significantly greater in the IVF than in ICSI groups. The completed hatching rate significantly increased with ZT in both groups. The maximum diameters of the completely hatched blastocysts were significantly smaller in the ZT than in non-ZT groups. The estimated TE cell numbers increased from hatching commencement to their maximum expansion points. The incompletely hatched ICSI-derived blastocysts intermittently herniated cells via small slits until degeneration. The completed hatching rate significantly decreased by the formation of ICSI penetration trace on zona pellucida of IVF-derived blastocyst.

Conclusion ICSI-derived blastocysts intermittently release proliferating cells and extracted TE cells and/or inner cell masses via a small slit; thus, blastocyst expansion is not sufficiently increased, leading to a reduced complete hatching rate. Therefore, the ICSI penetration trace potentially has negative effects on blastocyst expansion process in vitro and is a risk factor for the failure of completed hatching.

Keywords Blastocyst · Hatching · Hydrostatic pressure · Intracytoplasmic sperm injection · Morphology · Zona pellucida

Introduction

The spontaneous hatching of an embryo is crucial for the successful establishment of pregnancy. Hatching involves cell proliferation and proper expansion of the blastocyst by intake of fluid into its blastocoel, which causes an increase in the internal hydrostatic pressure. Consequently, the trophoblast epithelium is stretched and blastocyst is enlarged while the zona pellucida is simultaneously thinned [1]. During this process, combinations of lysine proteases are produced by the trophectoderm (TE) and/or uterus to dissolute the zona pellucida [2]. Moreover, blastocyst-derived pro- and anti-inflammatory cytokines regulate hatching of blastocysts [3].

There are several types of spontaneous hatching patterns [4, 5]. Assisted reproductive technology procedures, such as in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI), affect the hatching patterns [6]. During normal fertilisation, the sperm initiates the acrosome reaction and prevents polyspermy by inducing changes in the zona pellucida structure [7–9]. Moreover, a series of calcium oscillation are

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induced, which initiates oocyte activation and embryo development [10]. Although the ICSI procedure bypasses the zona pellucida and directly injects the sperm into the oocyte, the outer surface architecture of the blastocyst zona pellucida (spongy-like or smooth) is not correlated with the fertilisation method (IVF or ICSI) [11]. Previous studies have shown that the rate of complete hatching is lower in ICSI-derived blastocysts than in IVF-derived blastocysts until day 6 [6, 12]; however, it remains unclear whether incompletely hatched blastocysts can escape after day 6. The ICSI procedure is performed for infertility treatments worldwide; therefore, investigating the effect of ICSI on embryos/blastocysts in vitro is crucial.

The assisted hatching (AH) procedure helps in inducing embryo hatching. The zona pellucida is penetrated, melted or thinned by mechanical, laser beam or chemical methods [13]. Several studies have reported on the effect of the thinning range of the zona pellucida [14–16], but the results of studies on laser-assisted zona thinning (ZT) in ICSI-derived blastocyst and pregnancy outcomes are ambiguous [17–19]. Moreover, few reports have discussed the effect of ZT on the entire process.

Time-lapse monitoring is suitable to assess dynamic morphological processes, such as cleavage, embryo development and blastulation. Several events, including direct cleavage [20], reverse cleavage [21] and collapse [22] and embryo developmental parameters, including developmental speed [23] and expansion size [24], were analysed using time-lapse monitoring. In this study, time-lapse monitoring was used to assess the effect of the ICSI procedure on embryo hatching and to visualise the effects of ZT on the embryo.

Material and methods

Blastocysts

Blastocysts, which were obtained from patients who had a baby, were cryopreserved by the vitrification method [25] (Vitrification Media; Kitazato Corporation, Shizuoka, Japan). The mean (±standard deviation) age of women was 34.4 ± 3.9 (range, 25–41) years when the ovum pickup was performed. The blastocyst morphological score was determined according to the Gardner and Schoolcraft criteria [26] before cryopreservation. All procedures were performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients. This study was approved by the Umeda Fertility Clinic Institutional Review Board (171114).

Blastocyst warming, cell culture and time-lapse monitoring

The vitrified blastocysts were warmed according to the procedures described by Kuwayama [25] (Warming Media; Kitazato Corporation). After warming, blastocysts were placed into individual wells in a Geri® dish (Genea Biomedx, Sydney, NSW, Australia) in a time-lapse incubator (Geri+; Genea Biomedx) and cultured (CO_2, 6%; O_2, 5% at 37 °C and 80 ± 20% humidity) in an 80-μL drop of Geri Medium (Gems: Genea Biomedx) covered with 4 mL of light mineral oil (Oil for Embryo Culture; Irvine Scientific, Santa Ana, CA, USA) on a Geri® dish for 65–135 h (until blastocyst degeneration). Images were recorded every 5 min. The spontaneous hatching pattern was categorised according to the analysis by Kirkegaard et al. [6]. In brief, type A hatching pattern was characterised by an initial penetration of small TE projections (Fig. 1a), and type B hatching pattern was characterised by a sudden large rupture of the zona pellucida, followed by fast extrusion of the blastocyst (Fig. 1b).

The blastocyst diameter was measured at three time points: once at the point of post-warming, once during the hatching commencement and once at the point of maximum expansion until the establishment of completed hatching or degeneration. The ratio of the blastocyst diameter with the base diameter of the Geri® dish well (430 μm) was measured using Image J software (National Institutes of Health, Bethesda, MD, USA). The time of hatching commencement and completion were measured from at point of re-expansion and from at point of hatching commencement, respectively.

Assisted zona pellucida thinning

The ZT zone was determined based on previous studies [14, 16]. Half of the ZT was performed by thinning the zona pellucida at a depth of 54.9% ± 12.1% of the zona pellucida thickness; laser thinning was initiated at the 12 o'clock position and consecutive irradiations were generated until the 6 o'clock position (half thinning) was reached using the OCTAX Lasers & Imaging system (OCTAX microscience GmbH, Bruckberg, Germany).

Calculation of estimated trophectoderm cell numbers

The estimated number of TE cells was analysed according to Lagalla et al. [27]. Five to ten TE cell areas in each blastocyst at re-expansion, at hatching commencement and at the maximum expansion, were analysed using ImageJ software, and the average area of TE cells was calculated. It was hypothesised that the layer comprised similar-sized cells. The blastocyst surface area was calculated as $S = 4 \times \pi r^2$ at re-expansion, at hatching commencement and at the maximum expansion. The estimated number of TE cells was calculated by dividing the blastocyst surface area by the average TE cell area, and the estimated number excluded cells located outside the zona pellucida.

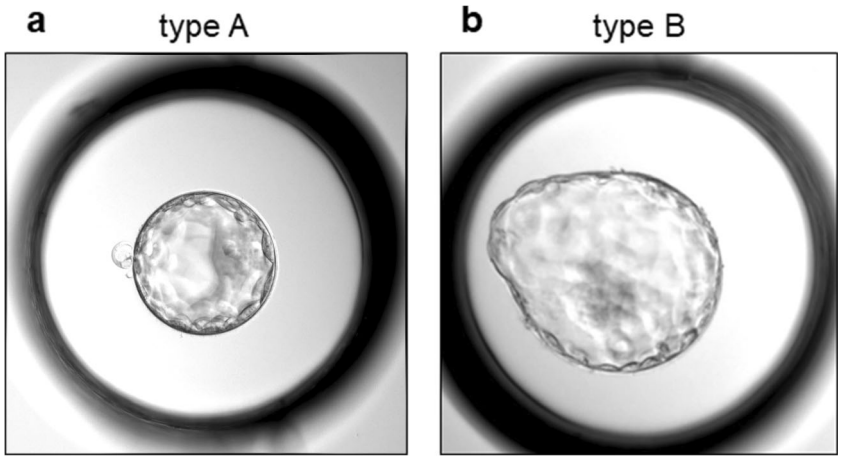

Fig. 1 Commencement of hatching patterns. **a** Type A is characterised by an initial penetration of small trophectoderm projections. **b** Type B is characterised by a sudden large rupture of the zona pellucida followed by fast extrusion of the blastocyst

Establishment of mimic model of the zona pellucida following ICSI using IVF-derived blastocyst

To eliminate the impact of the zona pellucida hardening and architecture by the difference of insemination method on completed hatching rate, as a mimic model of the zona pellucida of ICSI-derived blastocyst, the zona pellucida of IVF-derived blastocyst during shrinkage was penetrated using a piezo micromanipulator (outer diameter of micropipette, 5.88 μm; Prime Tech Ltd., Ibaraki, Japan, piezo impact drive unit; PIEZO PMM4G, Prime Tech Ltd.) after warming. The establishment of mimic model of the zona pellucida using a piezo micromanipulator was performed to avoid the following risk factors observed in our preliminary experiment: (1) TE cells potentially received injury at the point of breakthrough on the zona pellucida using the conventional ICSI injection pipette and (2) TE cells adhered to the conventional ICSI injection pipette after breakthrough of the zona pellucida, and the penetration point was then filled with TE cells when the pipette was gently withdrawn. Additionally, uniformity in the sizes of ICSI penetration traces between conventional ICSI and piezo ICSI was ensured by using injection pipettes with same outer diameters (conventional ICSI 6 μm (COOK Medical, Bloomington, IN, USA)). Thus, we believed that the IVF-derived blastocyst after formation of ICSI penetration trace mimicked the ICSI-derived blastocyst.

Statistical analysis

Rates of hatching pattern, hatching commencement, completed hatching, blastocyst stage before vitrification, inner cell mass (ICM) grade and TE grade were evaluated using Fisher's exact probability test that is used when any expected frequency is less than 1 or 20% of expected frequencies are less than or equal to 5. Female age, blastocyst diameter, thickness of the zona pellucida and time of event, such as hatching commencement, were evaluated using the Student's *t* test or Welch's *t* test. The estimated number of TE cells was evaluated using paired *t* test or Wilcoxon signed rank test. Moreover, relationship amongst completed hatching and insemination methods, female age, blastocyst stage, ICM and TE grades, thickness of the zona pellucida, blastocyst diameters and the estimated number of TE cells after warming, at commencement and at maximum expansion, were analysed using multiple logistic regression analysis. The confounding factors were omitted in order from the factor with the largest *p* value after multiple logistic regression analysis. Statistical analysis was then repeatedly performed until *p* values of all confounding factors became < 0.05. Data are presented as mean ± standard deviation. $p < 0.05$ was considered statistically significant.

Results

The blastocyst diameter at hatching commencement was significantly lower in type A than in type B hatching patterns ($p < 0.001$; Table 1). The time of hatching commencement from re-expansion was significantly shorter in type A than in type B ($p < 0.001$; Table 1). The rate of hatching commencement did not significantly differ between the IVF and ICSI groups (85.7% vs. 94.6%, respectively). However, hatching patterns differed between the groups (type A 25.0% vs. 92.5%, $p < 0.001$; type B 75.0% vs. 7.5%; $p < 0.001$, respectively; Table 2). The blastocyst diameter at hatching commencement was higher in the IVF group than in the ICSI group for both type A and B hatching patterns ($p < 0.05$ and $p < 0.05$, respectively; Table 2). The time of hatching commencement from re-expansion in both types did not significantly differ between IVF and ICSI groups (Table 2).

Next, the relationship between the insemination method and the rate of completed hatching of blastocyst was investigated. Blastocyst backgrounds, excepting female age, did not significantly differ between the IVF and ICSI groups (Table 3). The rate of completed hatching was significantly higher in the IVF group than in the ICSI groups (67.9% vs.

Table 1 Relationship between hatching pattern and blastocyst diameter at hatching commencement

	Hatching pattern		*p* value
	Type A	Type B	
No. of hatching blastocysts	61 (60.4%)	40 (39.6%)	
Blastocyst diameter at hatching commencement (μm)[a]	203.2 ± 17.8	260.1 ± 38.6	1.21E-11
Time of hatching commencement from re-expansion (min)[a]	462.1 ± 366.8	747.3 ± 389.6	0.0004

Type A: initial penetration of small trophectoderm projections; type B: sudden large rupture of the zona pellucida followed by fast extrusion of the blastocyst. Values are presented as numbers (%). Data are presented as mean ± standard deviation. $p < 0.05$ was considered significant

[a] Welch's *t* test

23.2%, respectively; $p < 0.001$), whereas that of incomplete hatching was significantly lower in the IVF group than in the ICSI groups (17.9% vs. 71.4%; $p < 0.001$; Table 3). These results suggest that the insemination method affects the hatching patterns and rate of completed hatching.

The diameters at hatching commencement and at the maximum expansion of completed hatching blastocysts were higher in the IVF group than in the ICSI group ($p < 0.001$ and $p = 0.002$, respectively; Table 3). Maximum expansion diameters of completely hatched blastocysts in both IVF and ICSI groups were higher than those of incompletely hatched blastocysts ($p = 0.048$ and $p = 0.022$, respectively; Table 3). These results suggest that there was not sufficient ICSI-derived blastocyst expansion, which led to a reduced completed hatching rate. Moreover, the zona pellucida of 28.6% ICSI-derived blastocysts with type A hatching pattern clearly displayed a small slit that may have been caused by a trace penetration following ICSI, and some TE cells herniated through the slit (Fig. 2a–f). In addition, the estimated TE cell numbers in blastocysts, excluding ICSI-derived blastocyst with type B hatching patterns, significantly increased from those at hatching commencement to at their maximum expansion points (Fig. 2g).

The completed hatching rate of ICSI-derived blastocysts was attempted to be improved using assisted ZT. The rate of completed hatchings significantly improved with ZT in both IVF and ICSI groups (IVF-ZT vs. IVF, 89.7% vs. 67.9%, $p = 0.004$; ICSI-ZT vs. ICSI, 77.8% vs. 23.2%, $p < 0.001$, respectively; Table 3). The maximum expansion diameters of completely hatched blastocysts were significantly smaller in the ZT groups than in the non-ZT groups (IVF; $p < 0.001$ and ICSI; $p < 0.001$, respectively; Table 3). These data suggest that completed hatching occurred when the blastocyst was expanded to a smaller degree because the zona pellucida was thin. In ICSI-derived blastocysts, the rate of completed hatching with contraction was significantly increased with ZT ($p = 0.014$; Table 3).

The hatching patterns of completed hatchings included types B and A + B, regardless of the insemination methods or ZT used; no completed hatchings were type A alone (Table 3). In incompletely hatched ICSI-derived blastocysts,

Table 2 Relationship between fertilisation methods and hatching commencement

	IVF	ICSI	*p* value
No. of warmed blastocysts	56	56	
No. of blastocysts at hatching commencement[a]	48 (85.7%)	53 (94.6%)	0.101
Hatching pattern[a]			
Type A	12 (25.0%)	49 (92.5%)	9.20E-13
Type B	36 (75.0%)	4 (7.5%)	9.20E-13
Blastocyst diameter at hatching commencement (μm)[b]	252.5 ± 39.5	201.5 ± 17.9	1.29E-12
Type A	213.6 ± 18.1	200.6 ± 17.0	0.038
Type B	265.5 ± 36.0	211.9 ± 27.8	0.024
Time of hatching commencement from re-expansion (min)[b]			
Type A	566.3 ± 511.5	436.6 ± 323.8	0.417
Type B	752.5 ± 401.3	700.0 ± 302.4	0.802

Type A: initial penetration of small trophectoderm projections, type B: sudden large rupture of the zona pellucida followed by fast extrusion of the blastocyst. Values are presented as numbers (%). Data are presented as mean ± standard deviation. $p < 0.05$ was considered significant

IVF in vitro fertilisation, *ICSI* intracytoplasmic sperm injection

[a] Fisher's exact probability test

[b] Student's *t* test or Welch's *t* test

Table 3 Relationship amongst fertilisation methods, assisted hatching procedure and completed hatching

	IVF	ICSI	IVF-ZT	ICSI-ZT
Female age (years)[a]	35.9 ± 3.6*	33.6 ± 4.1*	35.1 ± 2.5$^{\#\#}$	32.4 ± 3.5$^{\#\#}$
No. of warmed blastocysts[b]	56	56	58	54
Stage 3	35 (62.5%)	26 (46.4%)	27 (46.6%)	24 (44.4%)
Stage 4	21 (37.5%)	30 (53.6%)	31 (53.4%)	30 (55.6%)
Inner cell mass grade[b]				
No. of grade A	6 (10.7%)	4 (7.1%)	4 (6.9%)	1 (1.9%)
No. of grade B	25 (44.6%)	25 (44.6%)	31 (53.4%)	28 (51.9%)
No. of grade C	25 (44.6%)	27 (48.2%)	23 (39.7%)	25 (46.3%)
Trophectoderm grade[b]				
No. of grade A	4 (7.1%)	0 (0.0%)	1 (1.7%)	0 (0.0%)
No. of grade B	25 (44.6%)	24 (42.9%)	18 (31.0%)	24 (44.4%)
No. of grade C	27 (48.2%)$^{\$}$	32 (57.1%)	39 (67.2%)$^{\$}$	30 (55.6%)
Estimated no. of trophectoderm at re-expansion[a]	146.3 ± 41.9	160.9 ± 53.9	136.8 ± 35.3	148.9 ± 31.2
Blastocyst diameter after warming (μm)[a]	172.3 ± 14.3	167.9 ± 9.2	172.4 ± 14.5	168.1 ± 9.8
Thickness of zona pellucida (μm)[a]	11.0 ± 3.1	11.7 ± 3.3	11.3 ± 2.8	11.7 ± 2.2
Thickness of zona pellucida after ZT (μm)[a]	–	–	5.1 ± 1.4	5.1 ± 1.8
No. of hatching commencement blastocysts[b]	48 (85.7%)	53 (94.6%)	55 (94.8%)	53 (98.1%)
No. of completely hatched blastocysts[b]	38 (67.9%)**$^{,\$\$}$	13 (23.2%)**,††	52 (89.7%)$^{\$\$}$	42 (77.8%)††
No. of incompletely hatched blastocysts[b]	10 (17.9%)**	40 (71.4%)**,††	3 (5.2%)$^{\#}$	11 (20.4%)$^{\dagger\dagger,\#}$
No. of degeneration[b]	8 (14.3%)	3 (5.4%)	3 (5.2%)	1 (1.9%)
Blastocyst diameter at hatching commencement (μm)[a]	252.5 ± 39.5**$^{,\$\$}$	201.5 ± 17.9**	231.7 ± 27.7$^{\$\$,\#\#}$	207.7 ± 17.2$^{\#\#}$
Maximum diameter of hatching blastocyst (μm)[a]	265.2 ± 32.7**$^{,\$\$}$	228.1 ± 18.6**,††	236.9 ± 28.9$^{\$\$,\#\#}$	215.8 ± 17.7$^{\dagger\dagger,\#\#}$
Completely hatched groups				
Blastocyst diameter at hatching commencement (μm)[a]	258.8 ± 39.7**$^{,\$\$,\S}$	201.8 ± 19.3**	234.0 ± 26.3$^{\$\$,\#\#,\S\S}$	207.8 ± 17.7$^{\#\#}$
Maximum diameter of hatching blastocyst (μm)[a]	269.9 ± 33.6**$^{,\$\$,\ddagger}$	238.2 ± 20.6**,††,‡	239.2 ± 27.9$^{\$\$,\ \#\#,\ddagger\ddagger}$	215.8 ± 18.1$^{\dagger\dagger,\#\#}$
Incompletely hatched groups				
Blastocyst diameter at hatching commencement (μm)[a]	228.5 ± 29.3*,§	201.4 ± 17.6*	190.8 ± 17.7§§	207.4 ± 15.8
Maximum diameter of hatching blastocyst (μm)[a]	247.1 ± 22.2**$^{,\$\$,\ddagger}$	224.8 ± 16.9**,‡	197.7 ± 13.9$^{\$\$,\ddagger\ddagger}$	218.6 ± 16.4
Completely hatched pattern[b]				
Type A	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Type B	33 (86.8%)	4 (30.8%)	48 (92.3%)	30 (71.4%)
Type A + B	5 (13.2%)	9 (69.2%)	4 (7.7%)	12 (28.6%)
Time of type B hatching from re-expansion (min)[a]				
Type B	717.9 ± 398.7¶	700.0 ± 302.4¶	637.9 ± 415.3	600.2 ± 357.7¶
Type A + B	1154.0 ± 479.5¶	1310.6 ± 493.7¶,†	671.3 ± 206.5	848.3 ± 320.9¶,†
Completed hatching with contraction[b]	19 (50%)	7 (53.8%)†	19 (36.5%)$^{\#\#}$	37 (88.1%)$^{\dagger,\#\#}$
Completed hatching without contraction[b]	19 (50%)	6 (46.2%)†	33 (63.5%)$^{\#\#}$	5 (11.9%)$^{\dagger,\#\#}$
Time of hatching commencement to completed hatching (min)[a]				
Completed hatching with contraction	558.7 ± 413.5	377.0 ± 127.8	892.1 ± 742.1$^{\#}$	476.1 ± 348.4$^{\#}$
Completed hatching without contraction	440.3 ± 374.7	568.8 ± 333.4	565.6 ± 441.4$^{\#}$	317 ± 114.4$^{\#}$
Incompletely hatched pattern				
Type A	7 (70.0%)	38 (95.0%)	0 (0.0%)	7 (63.6%)
Type B	3 (30.0%)	0 (0.0%)	3 (100.0%)	1 (9.1%)
Type A + B	0 (0.0%)	2 (5.0%)	0 (0.0%)	3 (27.3%)

Type A: initial penetration of small trophectoderm projections, type B: sudden large rupture of the zona pellucida followed by fast extrusion of the blastocyst. Values are presented as numbers (%). Data are presented as mean ± standard deviation. $p < 0.05$ was considered statistically significant

IVF in vitro fertilisation, *ICSI* intracytoplasmic sperm injection, *ZT* Zona thinning

[a] Student's *t* test or Welch's *t* test

[b] Fisher's exact probability test

$^{**}p < 0.01$, $^{*}p < 0.05$, IVF vs. ICSI

$^{\$\$}p < 0.01$, $^{\$}p < 0.05$, IVF vs. IVF-ZT

$^{\dagger\dagger}p < 0.01$, $^{\dagger}p < 0.05$, ICSI vs. ICSI-ZT

$^{\#\#}p < 0.01$, $^{\#}p < 0.05$, IVF-ZT vs. ICSI-ZT

$^{\S\S}p < 0.01$, $^{\S}p < 0.05$, completely vs. incompletely hatched blastocysts at hatching commencement

$^{\ddagger\ddagger}p < 0.01$, $^{\ddagger}p < 0.05$, completely vs. incompletely hatched blastocysts at the maximum diameter

$^{\P}p < 0.05$, type B vs. type A + B

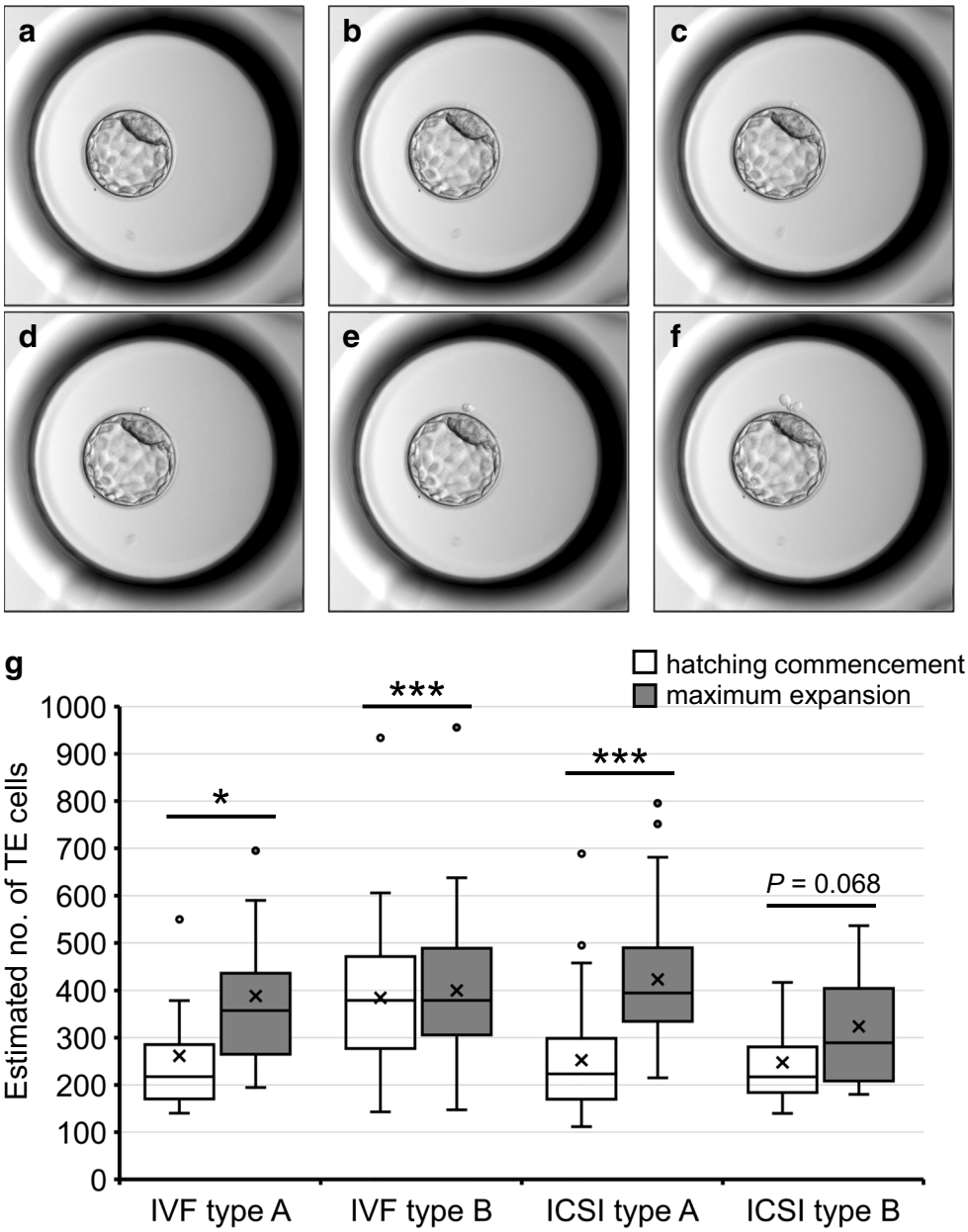

Fig. 2 The small slit on the zona pellucida of an intracytoplasmic sperm injection (ICSI)-derived blastocyst. **a**–**c** The slit appeared and was observed during expansion. **d**–**f** Certain trophectoderm (TE) cells herniated through the slit. **g** Estimated number of TE cells in blastocysts. The estimated number of TE cells was evaluated using paired *t* test or Wilcoxon signed rank test; data are presented as mean ± standard deviation. $p < 0.05$ was considered statistically significant. ***$p < 0.001$. *$p < 0.05$

TE cells and ICMs were intermittently herniated via small slits until degeneration (Fig. 3a–h). Several blastocysts with type A + B hatching pattern were initiated by type A hatching of small TE projections (Fig. 3i–k). Consequently, the completed hatching occurred on the other part of the blastocyst via the type B hatching pattern; simultaneously, a portion of the cells that penetrated the zona pellucida was removed (Fig. 3l–p). The time of type B hatching from re-expansion, excluding the IVF-ZT group, was significantly more in type A + B pattern than in type B pattern (Table 3). Additionally, the time of hatching commencement to completed hatching did not significantly differ regardless of the contraction of blastocyst during zona pellucida rupture (Table 3). Factors associated with completed hatching were insemination method, thickness of the zona pellucida, the maximum diameter of blastocyst and the estimated number of TE cells at maximum expansion (Table 4).

In addition, the effect of ICSI on completed hatching was investigated by making penetration trace on the zona pellucida of IVF-derived blastocyst. Hatching patterns differed between the IVF and mimic-ICSI groups (type A 7.7% (2/26) vs. 85.7% (24/28), $p < 0.001$; type B 92.3% (24/26) vs. 14.3% (4/28), $p < 0.001$, respectively; Fig. 4a, b). The rate of hatching commencement did not significantly differ between the IVF and mimic-ICSI groups (81.3% (26/32) vs. 87.5% (28/32); Fig. 4c), whereas that of completed hatching was significantly higher in the IVF than in the mimic-ICSI group (75.0% (24/32) vs. 31.3% (10/32), $p < 0.001$; Fig. 4d).

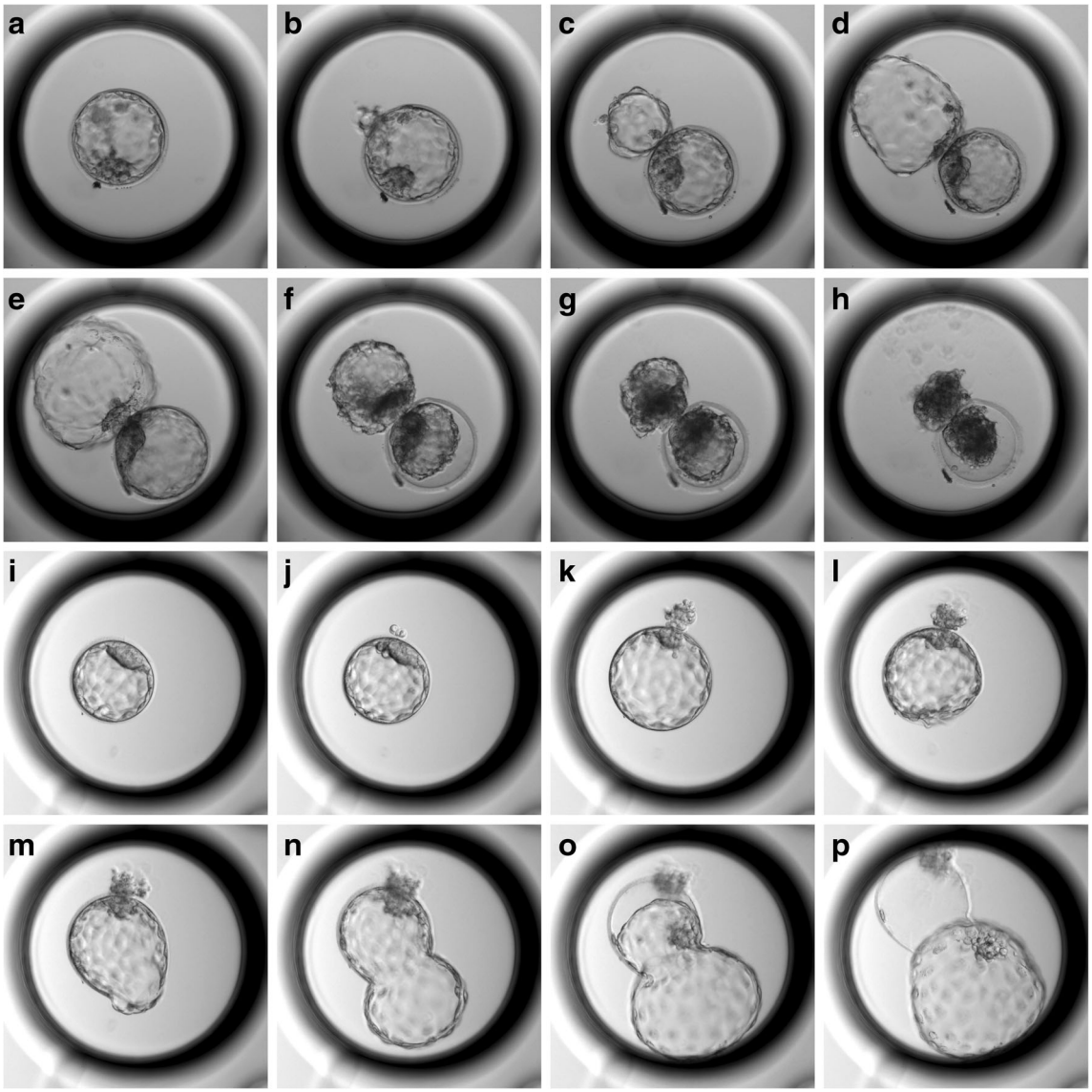

Fig. 3 Effect of ICSI on blastocyst hatching. **a**–**c** Type A blastocyst hatching was initiated by small trophectoderm projections. **d**–**h** However, completed hatching was not established, and the blastocyst degenerated. **i**–**k** Type A blastocyst hatching was initiated by small trophectoderm projections. **l**–**p** The completed hatching occurred on the other part of the blastocyst via the type B hatching pattern; simultaneously, a portion of cells that penetrated the zona pellucida was removed

Table 4 Associated factors of completed hatching

	β	Std. Error	p value	Odds ratio	95% CI
Intracytoplasmic sperm injection	− 1.278	0.608	0.035	0.279	0.085–0.916
Thickness of the zona pellucida	− 0.201	0.098	0.041	0.818	0.675–0.992
Maximum diameter of blastocyst	0.045	0.016	0.003	1.046	1.015–1.079
Estimated no. of trophectoderm at maximum expansion	− 0.007	0.002	0.003	0.993	0.988–0.998

Relationship amongst completed hatching and insemination methods, female age, blastocyst stage, ICM and TE grades, thickness of the zona pellucida, blastocyst diameters and the estimated number of TE cells after warming, at commencement and at maximum expansion were analysed using multiple logistic regression analysis

β regression coefficient, *CI* confidence interval

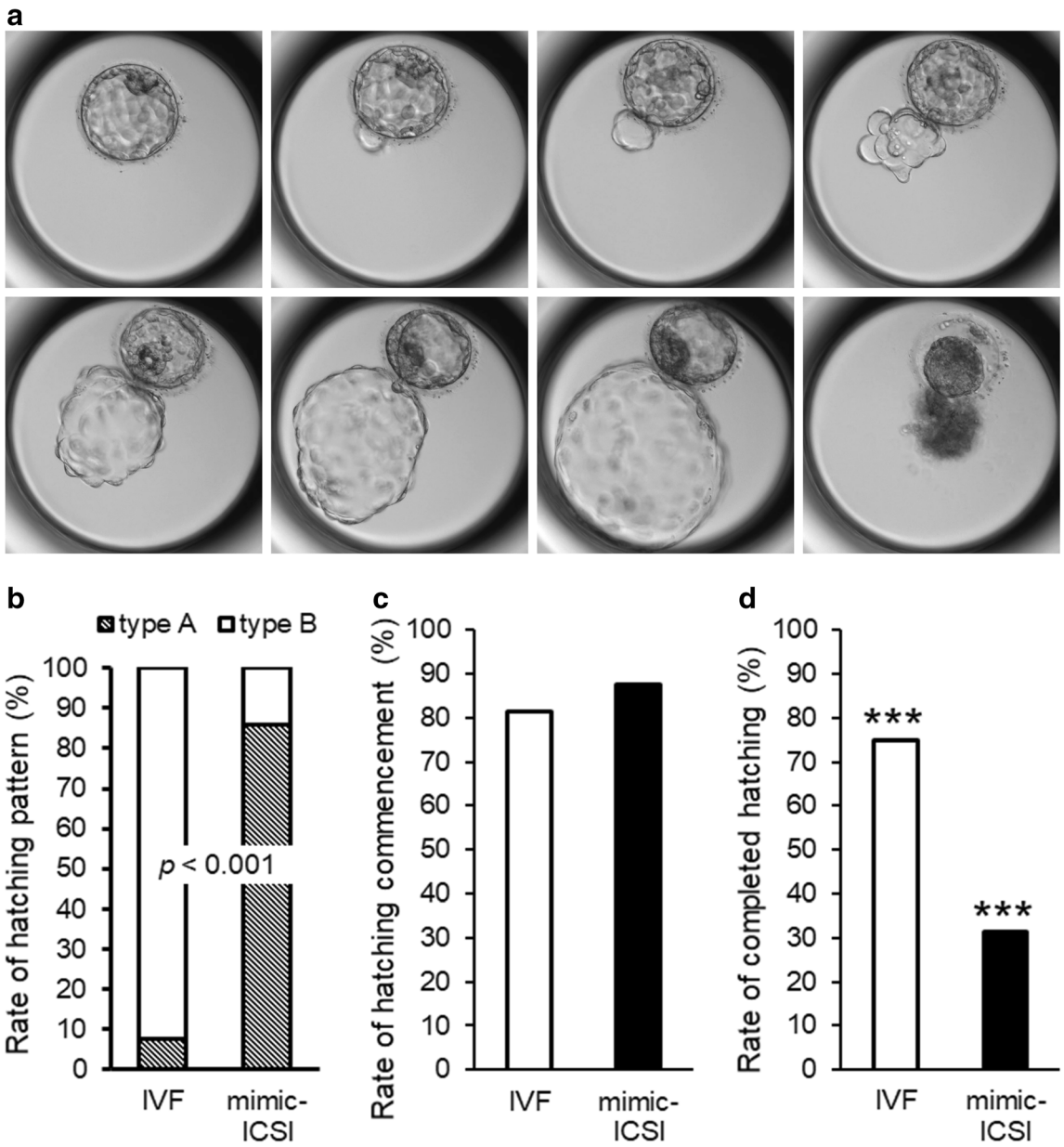

Fig. 4 **a** The hatching of mimic-ICSI model. **b** Rate of hatching pattern. **c** Rate of hatching commencement. **d** Rate of completed hatching. Rates of hatching pattern, hatching commencement and completed hatching were evaluated using Fisher's exact probability test between the IVF and mimic-ICSI groups. $p < 0.05$ was considered statistically significant. ***$p < 0.001$

Discussion

This study demonstrated that ICSI-derived vitrified–warmed blastocysts do not sufficiently expand even if hatching begins, and the rate of completed hatchings is lower than that observed under different circumstances. The rate of completed hatchings can be improved by performing a laser-assisted ZT procedure. Moreover, incompletely hatched ICSI-derived blastocysts displayed intermittently proliferating cells and herniated TE cells and/or ICMs until their degeneration; thus, hatching was not established in these blastocysts. Our results suggest that ICSI-derived blastocysts do not increase their internal hydrostatic pressure enough despite ongoing cell proliferation because of the presence of a small slit; consequently, blastocysts cannot expand enough and cannot normally hatch. The laser-assisted ZT procedure improved the rate of completed hatching by establishing hatchings with less expansion and lower internal hydrostatic pressures in the blastocysts.

Our results show that hatching patterns are associated with the blastocyst diameters at hatching commencement and time

of hatching commencement from re-expansion. Type A blastocyst diameter was significantly smaller than type B blastocyst diameter, and the time of hatching commencement from re-expansion was significantly shorter in type A than in type B. Blastocyst size increases as the developing stage progresses [28]. Accordingly, our results suggest that type A hatching initiates during the early stages of blastocyst expansion before reaching degree 5 (Gardner and Schoolcraft criteria, [26].

This study showed that the fertilisation method affects the commencement of hatching patterns: 92.5% of ICSI-derived blastocysts began to herniate the TE cells during the small size blastocyst stage, and 75.0% of IVF-derived blastocysts began to extrude via the sudden large rupture of the zona pellucida. Our findings of vitrified–warmed blastocysts are supported by the results of Kirkegaard et al. [6] who showed that fresh blastocyst hatching pattern was associated with fertilisation method. Although a previous study has demonstrated that cryopreservation steps address alterations in the zona pellucida, thereby causing irreversible damage on the further developmental competence of bovine embryo [29], we speculated that vitrification procedure exhibited no effect on the relationship between insemination methods and hatching pattern in the present study. Additionally, regarding of the relationship between hatching process and vitrification, previous study demonstrated that vitrification at the mouse eight-cell embryo stage increase the number of contractions of the pre-hatching blastocyst. However, completed hatching rate is reportedly not affected by vitrification [30]. Thus, we speculated that vitrification did not affect the completed hatching rate in this study.

Moreover, the difference of insemination methods potentially affects the zona pellucida hardening and architecture; the cumulus removal techniques, such as hyaluronidase and mechanical stripping, cortical granule reaction during ICSI procedure [31, 32] and multiple sperm exposure during IVF procedure [33], could further affect the zona pellucida hardening and architecture. However, the outer surface architecture of the blastocyst zona pellucida was not correlated with the fertilisation methods [11]. Additionally, the birefringence parameter as an indicator of human zona hardness was not different between IVF- and ICSI-derived embryos [34]. Accordingly, we speculated that zona hardness and architecture were not affected by insemination methods in the present study.

Further, we focused on the penetration point on zona pellucida following ICSI. Schwartz et al. [35] have reported that at 15 min post ICSI, the penetration site in either a spongy or a compact zona was barely detectable. Moreover, a previous study has shown that an isolated unusual hole in the zona pellucida of human ICSI pronuclear eggs and early embryos possibly corresponds to the residual gap through which sperms were introduced [11]. Our data showed that the zona pellucida of 28.6% ICSI-derived blastocysts evidently displayed a small slit that may represent the penetration point following ICSI (Fig. 2). Additionally, the rate of type A hatching pattern increased in the mimic-ICSI group. Accordingly, it was speculated that type A hatching pattern was mainly caused by the penetration of the zona pellucida during sperm injection, which is unique to ICSI.

The present study showed that although the rate of hatching commencement did not differ between IVF- and ICSI-derived blastocysts, the rate of completed hatching was significantly lower in the ICSI group than in the IVF group. These data revealed that the insemination method affects the hatching pattern, diameter of blastocyst expansion and completed hatching rate. This in vitro study showed that the completed hatching rate in the IVF-derived blastocyst was approximately threefold higher than that in the ICSI-derived blastocyst. However, pregnancy/implantation rates reportedly do not differ between IVF- and ICSI-derived embryos [36–38]. Our results suggest that there is a difference between the in vivo and in vitro hatching processes. During the in vivo hatching process, blastocyst–uterus interactions play a crucial role [2]. Accordingly, we speculate that lysine proteases, which are produced by the uterus to dissolute the zona pellucida, significantly affect the hatching process to assist the process by thinning of zona pellucida. Moreover, we hypothesised that the escape of blastocyst from zona pellucida is induced by complete dissolution of zona pellucida by proteases instead of the rupture of zona pellucida during the in vivo hatching process.

The diameter of maximum expansion of completely hatched blastocysts in both IVF and ICSI groups was greater than that of incompletely hatched blastocysts. Hatching involves cell proliferation and proper expansion of the blastocyst by the intake of fluid into the blastocoel, which causes an increase in the internal hydrostatic pressure that stretches the trophoblast epithelium. This leads to enlargement of its volume, consequently thinning the zona pellucida [1]. Our results suggest that incompletely hatched IVF- and ICSI-derived blastocysts did not sufficiently expand and consequently could not establish completed hatching.

In incompletely hatched ICSI-derived blastocysts, cell proliferation was observed: TE cells and/or ICMs were intermittently herniated via a small slit until degeneration (Fig. 3). Therefore, it was speculated that in ICSI-derived blastocysts, despite cell proliferation, the internal hydrostatic pressure causes TE cells and ICMs to flow out of the blastocyst through the small slit in the zona pellucida caused by the ICSI penetration point; consequently, hatching could not occur. In addition, the rate of incompletely hatched blastocyst increased in the mimic-ICSI group. Accordingly, our results suggest that ICSI penetration point is a risk factor for the failure of completed hatching.

Moreover, completed hatching patterns were only types B and A + B, regardless of the insemination method; type A alone did not establish completed hatching in this study. Accordingly, the type A pattern should be denoted as

'pseudo-hatching' rather than the initiation of hatching. The time of actual hatching (type B hatching) from re-expansion was significantly more in the type A + B group than in the type B group despite of the difference of insemination methods. This result suggested that in the presence of a small slit, the internal hydrostatic pressure takes time to increase causing the zona pellucida to rupture later.

Next, completed hatching rate of ICSI-derived blastocysts was attempted to be improved via laser-assisted ZT. Our results demonstrated that the completed hatching rate of ICSI-derived blastocysts was improved by ZT. The maximum expansion diameter of completely hatched blastocysts was significantly lower in the ZT groups than in the non-ZT groups. These data suggest that completed hatching resulted from reduced expansion and internal hydrostatic pressure in blastocysts following ZT. A previous study has shown that laser-assisted ZT does not facilitate blastocyst hatching by using superovulated-mated-derived mouse embryos [39]. Conversely, Blake et al. [40] reported that laser-assisted ZT increases the completed hatching rate. Their study supports our results. Accordingly, these data suggest that laser-assisted ZT improves the rate of embryo hatching in vitro.

A previous study has reported that the hatching process differs between in vivo and in vitro [41]. One limitation of this study is that blastocyst–uterus interactions during the hatching process could not be evaluated because frozen blastocysts that patients agreed to donate were used for all analyses. The second limitation is that the contribution of male factors [42] to blastocyst development/hatching processes remains unclear, although we eliminated the impact of male factor on their processes as much as possible by analysing the blastocyst form patients who had a baby and mimic-ICSI blastocysts. Although our results do not completely explain blastocyst hatching, they should be helpful in elucidating the hatching process. Moreover, the third limitation is the difficulty associated with performing AH for the all blastocysts because the TE cells in expanded blastocysts are directly in contact with zona at sites where the irradiation point of laser is in closer proximity for cases wherein the zona pellucida is thin. In fact, the decision of AH is dependent on the conditions of blastocyst after warming and flesh blastocysts in our clinic; in case of collapse, a quarter of the zona pellucida is breached, whereas in case of non-collapse, half of the zona pellucida is cautiously thinned and in case of an expanded blastocyst with thin zona pellucida, AH is not performed.

Conclusion

In summary, ICSI-derived vitrified–warmed human blastocysts intermittently release proliferating cells and extracted TE cells and/or inner cell masses via a small slit; therefore, the expansion of blastocysts does not sufficiently increase, leading to a reduced rate of complete hatching. Moreover, laser-assisted ZT induces hatching during the early stage of blastocyst expansion, ultimately leading to an improved rate of completely hatched blastocyst. Regardless of the difference in zona pellucida hardening and architecture due to the influence of insemination methods, completed hatching rate was found to decrease in the presence of ICSI penetration trace. To conclude, the ICSI penetration trace potentially has negative effects on blastocyst expansion process in vitro and is a risk factor for the failure of completed hatching.

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Author's contributions T. I. designed this study, collected and analysed data and drafted the manuscript. M. U contributed to the data analysis and critically reviewed the manuscript. K. M and Y. Y revised the manuscript. All authors approved the final manuscript.

Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Umeda Fertility Clinic Institutional Review Board (171114) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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

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