Outcomes of vitrified-warmed cleavage-stage embryo hatching after *in vitro* laser-assisted zona pellucida thinning in patients

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Abstract. The aim of the present study was to determine whether the size of the zona pellucida (ZP) thinning area by laser-assisted hatching affected the potential development of vitrified-warmed embryos. A total of 196 vitrified-warmed cleavage-stage embryos (from 49 patients, four sister embryos per patient) were used in the study, i.e., four sister embryos from each patient were randomly assigned to four groups: A control group of embryos that were not zona-manipulated (zona intact, group A); one experimental group of embryos in which a quarter of the zona pellucida was thinned using laser-assisted ZP thinning (group B); a second experimental group of embryos in which half of ZP was thinned (group C); and a third group in which two-thirds of the ZP was thinned (group D). Subsequent blastocyst development was assessed. Microscopy was performed to study the hatching process of the embryos after zona thinning. The blastocyst formation rates were 71.43% in group A, 67.35% in group B, 65.31% in group C, and 51.02% in group D (groups B-D vs. group A, P=0.661, P=0.515, P=0.038, respectively). The rates of complete hatching were 30.61% in group A, 38.78% in group B, 61.22% in group C, and 48.98% in group D (groups B-D vs. group A, P=0.396, P=0.002, P=0.063, respectively). For a subgroup of patients, there was a significant difference in the complete hatching in all the groups for women aged <35 years (P=0.011), and there was a significant difference in the complete hatching in all the groups for secondary infertility women (P=0.022). There was no significant difference in the blastocyst formation rates in the different groups of women aged ≥35 years (P=0.340). In addition, there was no significant difference in the complete hatching in the different groups among women aged ≥35 years (P=0.492). The results of the present study showed that in vitrified-warmed embryo transfers at the cleavage-stage, and the two-thirds zona pellucida thinning group demonstrated a significantly decreased blastocyst formation rate compared with the control group, while the half zona pellucida thinning group demonstrated a significantly increased complete hatching rate compared with the control group, which may have a high value in clinical application.

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Introduction

It is widely known that improving the embryo implantation rate is one of the main objectives of assisted reproductive technologies (ARTs). The most common method to achieve better results is to obtain and transfer multiple embryos. The surplus embryos produced using ARTs may be cryopreserved for subsequent use (1). The success of cryopreservation may undoubtedly increase the cumulative pregnancy rates of ARTs. However, these results appear to indicate a reduction in the clinical pregnancy and implantation rates compared with fresh embryos (2).

A glycoprotein layer, known as the zona pellucida (ZP), surrounds human embryos and permits only acrosome-intact sperm to fertilize the oocyte by blocking the entry of multiple sperm. After fertilization, the ZP compresses and shapes the embryo, protecting it from microorganisms and immune cells. At the blastocyst stage, the embryo breaks out of the ZP to begin the developmental process; failure at this stage can prevent implantation. Hatching of the embryo is a critical step in the sequence of physiological events that culminate in implantation. Failure to hatch, due to intrinsic abnormalities in the blastocyst or ZP, may be the main factors limiting human-assisted reproductive efficiency, and the effects of zona hardening on embryo hatching are probably one of the consequences of the process of freezing and thawing embryos (3). Therefore, artificial thinning of the zona or drilling of the zona may improve the embryo's potential and thus improve the clinical outcome of the thawing cycle. Since the 1980s, assisted hatching (AH) has been used to improve the chances of implantation during ARTs (1).

AH using a 1.48-μm diode laser yields a better outcome than AH using mechanical or chemical methods (4,5).

However, to the best of our knowledge, no single study has been able to demonstrate sufficient evidence favourable to AH, and the current research conclusions are not unanimous (6-13). In a large meta-analysis, Martins *et al* showed that laser-AH (LAH) is currently one of the best, safest and most effective AH methods (14). LAH can improve the clinical pregnancy rate of the thawing cycle. Since LAH can be divided into artificial thinning of the zona and drilling of the zona, previous findings have shown that the former is better than that the latter *in vitro* (15,16). Hiraoka *et al* have shown that vitrification can increase the hardness of zona, and the embryo implantation and clinical pregnancy rates in the half ZP thinning FET cycle was superior to that of a quarter ZP, although these studies lacked a control group (1). However, to the best of our knowledge, no study has included a sufficient sample to properly evaluate the effect of LAH on assisted reproduction outcomes (1,17). In the present study, we used vitrified frozen-thawed sister embryos and performed laser-assisted ZP thinning to reduce a quarter, a half and two-thirds area of the zona in the experimental groups.

The aim of the current study was to observe the rate of blastocyst formation and the complete hatching rate to determine which size of ZP thinning by LAH is optimal for embryonic development and to determine the best LAH method.

Materials and methods

Patients and embryos. The present study originally included 58 infertility patients who were admitted to the IVF unit of Linyi People's Hospital (Linyi, China) from May 1, 2011 to November 31, 2011, while 9 patients with <4 frozen-thawed embryos (grade I or II) were excluded. Among them, there were 26 women aged <35 years (13 in primarily infertility and 13 in secondary infertility) and 23 women aged ≥35 years (11 in primarily infertility and 12 in secondary infertility). All of the patients with frozen-thawed embryos had >4 high quality sister embryos surviving, and each couple had succeeded in delivering at least one child. Any unused embryos were discarded. The embryonic blastomeres selected dissolved ≤2 (from 49 IVF patients) frozen-thawed blastomeres on day 3, and grade I or II embryos at the 7-10 cell stage were selected. The embryos were scored as indicated in a previous study (18): grade I, uniform blastomere size; regular morphology; intact zona; homogeneous cytoplasm, clear, with no particle phenomenon; and embryo fragmentation of <5%. Grade II, slightly uneven blastomere size; slightly irregular morphology; cytoplasmic visible particle phenomenon; and debris of 6-20%. The sister embryos from the same patient were randomly divided into four groups: a control group of embryos that were not zona-manipulated (zona intact, group A); one experimental group of embryos in which a quarter area (1/4) of the ZP was thinned using laser-assisted ZP thinning (group B); a second experimental group of embryos in which a half area (1/2) of the ZP was thinned (group C); and a third group in which a two-thirds area (2/3) of the ZP was thinned (group D). Subsequent blastocyst development was assessed (Fig. 1). The [patients were included in the study if they met the following requirements: 24-39 years of age and a body mass index of 18-28 kg/m^2. The fecundities of the male partners of the patients were normal according to the World Health Organization criteria (19).

The present study was conducted at the Linyi People's Hospital (Shandong, China) and was approved by the Ethics Committee of Linyi People's Hospital. A written informed consent form was obtained from all patients.

The embryos, which were all at the 7-10 cell stage, were pooled and divided into four groups by defocusing the microscope to prevent any bias in the selection.

Warming of embryos and AH. Vitrification was performed on embryos derived from IVF or ICSI cycles. It has been shown that these two sources of embryos have no effect on their cultivation (20-22).

Over 50% of the blastomeres in one embryo survived and were continuously cultured. Otherwise, the embryos were discarded. Vitrified embryos considered grade I or II embryos were warmed in the following manner: the protective cover was warmed in liquid nitrogen, and the end of the polypropylene strip was immersed directly into 1 ml of 37˚C 1.0 mol/l sucrose solution for 1 min. The embryos were then transferred into 1 ml of 37˚C 0.5 mol/l sucrose solution for 3 min and washed twice in the base medium for 5 min. AH was performed using a previously described method (23). The average thickness of the zona pellucida was calculated by the numerical values measured at the 12, 3, 6, and 8 o'clock positions from the inside to the outside.

The laser system consisted of a Fertilase 1.48-μm laser, 100 mW, operated by an Octax Eyeware digital interface (MTG, Bruckberg, Germany) and positioned on a Nikon Eclipse TE2000-U inverted microscope (Nikon Instruments Europe B.V., Badhoevedorp, The Netherlands).

Embryos were randomly divided into four groups, and the embryos were thawed immediately after LAH. The frozen-thawed embryos were placed in the inverted microscope at 37˚C, and the embryos were fixed with a holding pipette at the 9 o'clock position. The thinning procedure was standardized during pilot experiments to establish a smooth lasered area. A 5-μm hole was formed in the zona pellucida using one laser shot. The laser thinning was initiated at the 12 o'clock position, and consecutive irradiations were performed untill the 3 o'clock position (quarter thinning, group B), the 6 o'clock position (half thinning, group C) or the 8 o'clock position (two-thirds thinning, group D) at a depth of 60-80% of the zona pellucida thickness (Fig. 2).

After LAH, the embryos were washed several times and transferred to G2 medium. The embryos were then cultured in an atmosphere of 6% CO_2, 5% O_2 and balance N_2, pH 7.32 at 37˚C using a Labotect C200 incubator (Labotect Labor-Technik-Göttingen GmbH, Göttingen, Germany). In the control group, the same conditions were performed directly after the embryos were thawed. Any manipulations were performed at room temperature (22-23˚C). In the present study, observations were performed on day 5, at 6:00 a.m. and in the afternoon. Complete blastocysts spreading (beyond a score of 4BB) was recorded four times. Observation was continually performed on day 6, at 7:00 a.m. and in the afternoon in order to record whether the blastocyst had completely hatched.

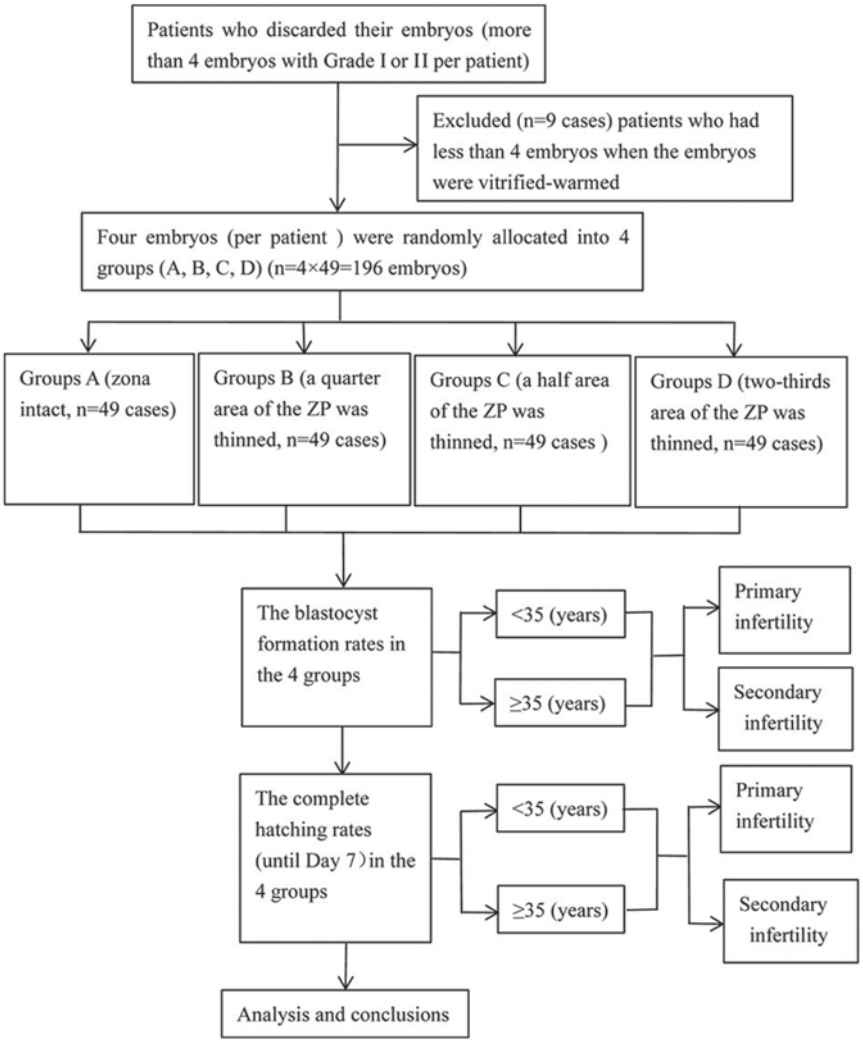

Figure 1. Flow diagram of embryos throughout each stage of the study.

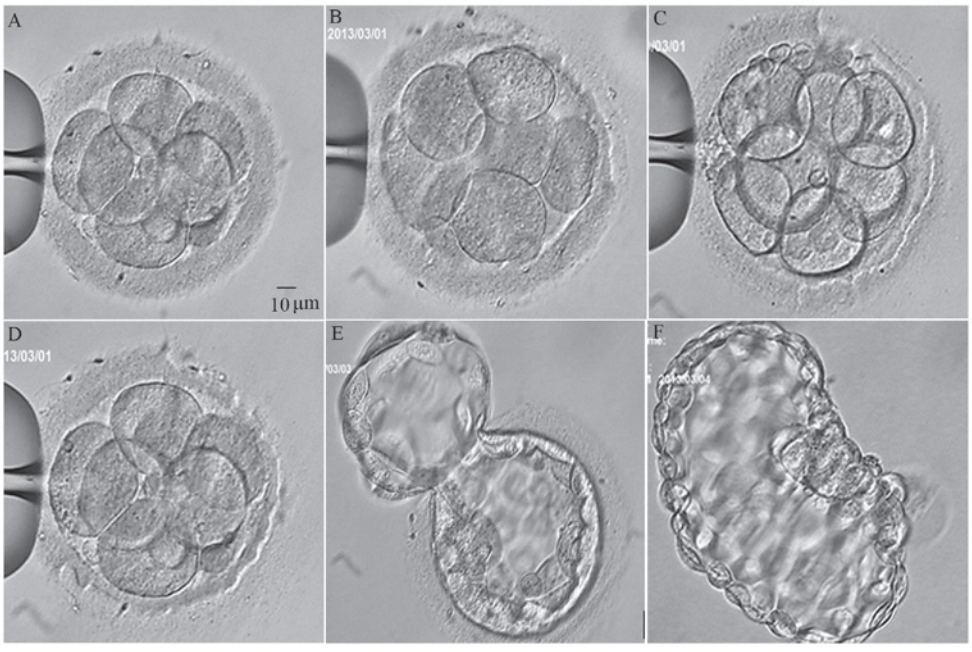

Figure 2. Vitrified-warmed human cleavage-stage embryos and the hatching of the embryos (A) stabilized with a holding pipette with the zona intact, (B) after quarter ZP thinning using laser-assisted hatching, (C) after half ZP thinning using laser-assisted hatching, (D) after two-thirds ZP thinning by laser-assisted hatching, (E) partial hatching of the embryos, and (F) complete hatching. Bar, 10 μm. ZP, zona pellucida.

Table I. Rate of blastocyst formations in the different groups.

Groups	No. of blastocyst formation	No. of blastocyst non-formation	Rate of blastocyst formation	P-value
Group A	35	14	71.43%	-
Group B	33	16	67.35%	0.661[a]
Group C	32	17	65.31%	0.515[b]
Group D	25	24	51.02%	0.038[c]

[a]Group B comparison with control group (group A) (67.35 vs. 71.43%). [b]Group C comparison with control group (group A) (65.31 vs. 71.43%). [c]Group D comparison with control group (group A) (51.02 vs. 71.43%). Values with P<0.05 were considered statistically significant.

Table II. Rate of complete hatching (until day 7) in the different groups.

Groups	No. of complete hatching	No. of no or partial hatching	Rate of complete hatching	P-value
Group A	15	34	30.61%	-
Group B	19	30	38.78%	0.396[a]
Group C	30	19	61.22%	0.002[b]
Group D	24	25	48.98%	0.063[c]

[a]Group B comparison with control group (group A) (38.78 vs. 30.61%). [b]Group C comparison with control group (group A) (61.22vs. 30.61%). [c]Group D comparison with control group (group A) (48.98 vs. 30.61%). Values with P<0.05 were considered statistically significant.

Statistical analysis. SPSS 23.0 software for Windows was used for statistical analysis (SPSS, Inc., Chicago, IL, USA). The data were reported as the mean ± standard deviation (SD). The differences in the variables between the groups were statistically analysed using the Student's t-test or one-way ANOVA. For the purpose of analysis of the categorical data (e.g., blastocyst formation rate, and hatching rate), significant differences were evaluated using the Chi-square test when appropriate. P<0.05 was considered statistically significant.

Results

Patient information. Fifty-eight patients were initially included in the study, and 9 patients with <4 frozen-thawed embryos (grade I or II) were excluded. A total of 196 embryos from 49 patients were eventually enrolled in the study.

The mean age for the <35-year women was 27.23±3.02 (mean ± SD) (95% CI, 26.00-28.45) and the mean age of the ≥35-year women was 36±1.17 (95% CI, 35.50-36.50) for the groups. The mean zona were 17.46±0.58, 17.35±0.51, 17.36±0.54 and 17.58±0.42 μm for groups A-D, respectively. There was no significant difference in the thickness of the four groups (P=0.088).

Rate of blastocyst formation and complete hatching. The blastocyst formation rates were 71.43% (35/49) in group A, 67.35% (33/49) in group B, 65.31% (32/49) in group C, and 51.02% (25/49) in group D [overall group (groups B-D) comparison with group A, P=0.661, P=0.515, P=0.038, respectively] (Table I). The rates of complete hatching were 30.61% (15/49) in group A, 38.78% (19/49) in group B, 61.22% (30/49) in group C, and 48.98% (24/49) in group D [comparison with group A, P=0.396 (group B vs. group A), P=0.002 (group C vs. group A), P=0.063 (group D vs. group A)] (Table II).

Blastocyst formation and complete hatching in the subgroups. The blastocyst formation rates for the subgroup of women aged <35 years were 73.08% (19/26) in group A, 69.23% (18/26) in group B, 80.77% (21/26) in group C, and 53.85% (14/26) in group D. There was no significant difference in the blastocyst formation rates in the different groups among women aged <35 years (χ^2=4.694, P=0.196). The blastocyst formation rates for the subgroup of patients aged ≥35 years were 69.57% (16/23) in group A, 65.22% (15/26) in group B, 47.83% (11/23) in group C, and 47.83% (11/23) in group D. There was no significant difference in the blastocyst formation rates in the different groups in women aged ≥35 years (χ^2=3.357, P=0.340) (Table III).

In addition, the rates of complete hatching (women aged <35 years) were 30.77% (8/26) in group A, 50% (13/26) in group B, 76.92% (20/26) in group C, and 53.85% (14/26) in group D. There was a significant difference in the complete hatching in the different groups among women aged <35 years (χ^2=11.230, P=0.011). The rates of complete hatching (women aged ≥35 years) were 30.43% (7/23) in group A, 26.09% (6/23) in group B, 43.48% (10/23) in group C, and 43.48% (10/23) in group D. There was no significant difference in the complete hatching in the different groups among women aged ≥35 years (χ^2=2.410, P=0.492) (Table IV).

Blastocyst formation and complete hatching in the subgroups of patients with primary and secondary infertility. The blastocyst formation rates for the subgroup of patients with primary infertility were 58.33% (14/24) in group A, 54.17% (13/24) in group B, 54.17% (13/24) in group C, and 33.33% (8/24) in group D. There was no significant difference in the blastocyst formation rates in the different groups with primary infertility (χ^2=3.667, P=0.300). The blastocyst formation rates for the subgroup of patients with secondary infertility was 84% (21/25) in group A, 80% (20/25) in group B, 76% (19/25) in group C, and 68% (17/25) in group D. There was no significant difference in the blastocyst formation rates in the different groups with secondary infertility (χ^2=1.976, P=0.577) (Table III).

In addition, the rates of complete hatching (primary infertility) were 20.83% (5/24) in group A, 33.33% (8/24) in group B, 45.83% (11/24) in group C, and 29.17% (7/24) in group D. There was no significant difference in the complete hatching in the different groups with primary infertility women (χ^2=3.573, P=0.311). The rates of complete hatching (secondary infertility) were 40% (10/25) in group A, 44% (11/25) in group B, 76% (19/25) in group C, and 68% (17/25) in group D. There was a significant difference in complete hatching in the different groups among women with secondary infertility women (χ^2=9.588, P=0.022) (Table IV).

Table III. Blastocyst formation in the different subgroups.

		No. of blastocyst formation		Failed to blastocyst formation		
Groups	Age (years)	Primary infertility	Secondary infertility	Primary infertility	Secondary infertility	Total
A	<35	8	11	5	2	26
	≥35	6	10	5	2	23
B	<35	8	10	5	3	26
	≥35	5	10	6	2	23
C	<35	9	12	4	1	26
	≥35	4	7	7	5	23
D	<35	5	9	8	4	26
	≥35	3	8	8	4	23

Table IV. Complete hatching (until day 7) in the different subgroups.

		No. of complete hatching		No. of no or partial hatching		
Groups	Age (years)	Primary infertility	Secondary infertility	Primary infertility	Secondary infertility	Total
A	<35	3	5	10	8	26
	≥35	2	5	9	7	23
B	<35	6	7	7	6	26
	≥35	2	4	9	8	23
C	<35	8	12	5	1	26
	≥35	3	7	8	5	23
D	<35	5	9	8	4	26
	≥35	2	8	9	4	23

Discussion

Despite the rapid development of IVF and ICSI, the implantation rate of embryos remains relatively low. It has been indicated that only 15% of embryos were successfully transplanted into the uterine cavity in the 1990s (24). Even if a normal chromosome embryo has good developmental potential, it may be unable to grow successfully because of failure to hatch (25). Embryo implantation is affected by numerous factors, including uterine endometrial receptivity, operation of transplantation technology, and embryo hatching ability (26). Selective application of LAH in ART may enhance hatching ability. Since hatching ability plays an important role in the process of embryonic development, the basic conditions that ensure the success of a hatched embryo include that the ZP exhibits good elasticity to become thin with the expansion of the blastocyst (27). Thus, a potential mechanism to improve embryo implantation ability may be technological assurance of embryos at an earlier stage of hatching and early contact with the endometriium. ZP thinning may accelerate nutrient exchange between the liquid culture and embryo, and may promote embryonic development and blastocyst formation (28,29).

Although vitrification technology has been previously developed, it has not been promoted. Studies on the effect of LAH on the cycle of FET have focused more on programme freezing (9). The vitrification process can increase embryo zona pellucida hardness and affect hatching compared with programmed freezing. Thus, patients who use frozen-thawed embryos benefit from LAH. Most of the current literature regarding LAH measures compares the embryo implantation and clinical pregnancy rates, and when the patients were grouped, the results were inevitably affected by endometrial receptivity and many other factors (30). Although most of the potential factors that interfere with the implantation of the embryo have been eliminated, the embryo implantation mechanism is extremely complex. In the present study, we established a control group and observed the effect of different ZP circumferences of LAH on the potential of embryonic development in order to select the optimal LAH method. The results showed that the two-third zona pellucida thinned group (group D) demonstrated a significantly decreased blastocyst formation rate compared with the control group (group A). In addition, the fully hatched rates of the blastocysts in group C (one-half zona thinning) were significantly higher than that of group A, while the remaining experimental groups showed no

significant difference compared with the control group. These results are consistent with those demonstrated in previous studies (1). In the subgroup of patients, there was a significant difference in the complete hatching in the different groups for women aged <35 years (P=0.011), and there was a significant difference in the complete hatching in the different groups of secondary infertility women (P=0.022).

The LAH drilling method is less time-consuming than the ZP thinning method. However, previous findings have shown that if the drilling is extremely small, it causes blastocyst hatching to be incarcerated. By contrast, when the drilling is extremely large, some blastomeres in the embryos may be lost before they are closely connected (31). Therefore, many centres favour zona pellucida thinning with AH. However, findings regarding the size of the thinned area of the zona pellucida are inconclusive. The reasons for this include, the difference in AH method and technology, the difference in experimental design, the difference in freezing method (programmed freezing or vitrification), and patient characteristics.

In the past, limited data were reported on the final results of the blastocysts *in vitro* after AH (27). In the present study, we employed vitrified-warmed sister embryos of grade I and II and performed zona thinning by ablating one-quarter, one-half or two-thirds of the ZP circumference. The embryos were cultured using an *in vitro* method to observe the embryonic development potential. The results of the present study showed that the blastocyst formation rates of the four groups were 71.43% (control group), 67.35% (quarter area zona thinning), 65.31% (half area zona thinning), and 51.02% (two-thirds area zona thinning). These results showed that the blastocyst formation rate in two-thirds area zona thinning group was lower than that in control group (P=0.038). The random grouping of sister embryos in the same patient was performed to eliminate the difference between individuals and may reflect the effect of the laser-assisted ZP thinning in the developmental potential of embryos. However, whether the different thinning areas in the sister embryos of the same patient influence the hatching rate remains to be determined. The current study elaborated that the one-half zona pellucida thinning method significantly improved the blastocyst completely hatched rate compared to the control group, particularly with women aged <35 years or in women with secondary infertility. This result may have a high value in clinical application. The data of the present study are small scale, and therefore more studies are required to confirm the results.

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