Number of biopsied trophectoderm cells is likely to affect the implantation potential of blastocysts with poor trophectoderm quality

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Objective: To evaluate whether the developmental potential of the blastocyst is affected by the number of trophectoderm (TE) cells biopsied in preimplantation genetic diagnosis (PGD) cycles.
Design: Retrospective study.
Setting: University-affiliated center.
Patient(s): Women underwent PGD cycles of blastocyst biopsy and fluorescence in situ hybridization analysis.
Intervention(s): Not applicable.
Main Outcome Measure(s): Biopsied TE cell number of blastocysts, survival, and implantation rates.
Result(s): The biopsied TE cell number was affected by the TE quality and experience of different embryologists. The diagnostic efficiency increased when from one to five cells were biopsied (86.7%, 91.7%%, 96.0%, 96.8%, to 98.7%) and was maximized when more than six cells were biopsied. To compare the clinical efficiencies, blastocysts were divided into four groups according to biopsied TE cell number: 1–5, 6–10, 11–15, and 16–41. For the blastocysts with grade A TE score, no significant difference was observed in the survival and implantation rates among the four groups. For the blastocysts with grades B and C TE scores, the survival rates showed no significant differences among the four groups, but a significant decreasing trend in implantation rates was observed with increasing biopsied TE cell number.
Conclusion(s): The implantation potential is negatively affected by the biopsied TE cell number in blastocysts with poor TE morphological score. (Fertil Steril® 2016;105:1222–7. ©2016 by American Society for Reproductive Medicine.)
Key Words: Blastocyst biopsy, biopsied cell number, trophectoderm quality, implantation

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Cleavage-stage embryo biopsy is widely used in preimplantation genetic diagnosis/screening (PGD/PGS) cycles (1). Although an early study reported that removal of one or two cells at the eight-cell stage did not adversely affect the further development of biopsied embryos in vitro (2), two late prospective trials showed that removal of two blastomeres significantly decreased the likelihood of blastocyst formation and implantation rate when compared with removal of one blastomere (3, 4), suggesting that the number of biopsied cells was critical in cleavage-stage embryo biopsy. Recently, some studies have shown that even the one-cell biopsy of cleavage-stage embryos significantly impairs embryonic implantation potential, while blastocyst biopsy does not (5, 6); these results indicate that blastocyst biopsy is safer than cleavage-stage embryo biopsy.

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The blastocyst stage is currently supposed to be an optimal time to perform biopsies for PGD/PGS (7); however, investigations to determine the appropriate number of biopsied trophectoderm (TE) cells in blastocyst biopsies are limited.

In a study performed by Dokras et al. (8), enumeration of the biopsied TE cells by Giemsa staining after donated blastocyst biopsies showed hCG secretion fell to low levels when more than 10 cells were removed. In many clinical studies applying blastocyst biopsy for PGD/PGS cycles, between four and 10 TE cells were biopsied according to the experience of the operator (9–12). However, the exact number of biopsied TE cells was hard to count visually because the cells are small and usually remain as a clump. In most studies using comparative genome hybridization or single-nucleotide polymorphism array technology for genetic testing (13–16), the biopsied TE cells were used for genome amplification and the cell number was impossible to know. Fluorescence in situ hybridization (FISH), which has been used in a few studies (17–19), allows enumeration of the biopsied TE cells after nuclear staining, but the exact cell numbers were not shown in these studies.

The impact of TE cell loss on blastocyst implantation potential depends on the total number of TE cells. The total cell number of blastocysts varies with the developmental stage and quality. Hardy et al. (20) showed that the mean TE cell number of day 5 blastocysts was 37.9 ± 6.0, while by day 7, the mean TE cell number was 89.6 ± 15.2. Another study from Fong et al. (21) showed that the total TE cell number for good-quality blastocysts was significantly greater than that for poor-quality blastocysts. It can be speculated that the damage to blastocyst developmental potential caused by TE biopsy would be less for blastocysts with a greater number of TE cells.

To evaluate the appropriate number of biopsied TE cells in PGD/PGS, the present study counted the biopsied TE cell number after blastocyst biopsy combined with FISH analysis, and the clinical outcomes were compared for blastocysts with different biopsied TE cell numbers.

MATERIALS AND METHODS

Patients and Clinical Protocols

In this study, patients who underwent PGD by FISH analysis were selected. These patients were usually young and could not afford the cost of DNA amplification-based comprehensive chromosomal analysis. In total, 589 couples (average female age, 28.76 ± 3.37 years; range, 21–39 years) underwent 638 PGD cycles (blastocyst biopsy combined with FISH analysis) at the Reproductive and Genetic Hospital of CITIC-Xiangya between September 2012 and November 2014. Among the 589 couples, 158 were Robertsonian translocation carriers, 315 were reciprocal translocation carriers, 65 were inversion carriers, 31 were sex chromosomal aneuploidy, two carried X-linked genetic disease, and 18 carried Y-chromosome microdeletions. This retrospective study was deemed exempt from ethical review by the Ethics Committee of the Reproductive and Genetic Hospital of CITIC-Xiangya.

The ovarian stimulation protocols were either a long luteal GnRH agonist protocol or an antagonist protocol, as described by Tan et al. (5). HCG (5,000–10,000 IU, Pregnyl; Merck) was injected when two-thirds of the follicles reached 18 mm. Oocyte retrieval was performed 34–36 hours later under ultrasound guidance.

Embryo Culture and TE Scoring

All oocytes were fertilized by intracytoplasmic sperm injection 4–6 hours after oocyte retrieval, and normal fertilization was identified 16–18 hours after injection by the presence of two pronuclei and two polar bodies. Embryos were cultured in sequential media (G1.5 and G2.5, Vitrolife) to blastocyst stage at 37°C under 6% CO_2, 5% O_2, and 89% N_2 in a COOK mini-incubator.

For the zona drilling, a 25-μm hole was made by laser in the zona pellucida of all embryos on day 3 after fertilization. On the morning of day 6, the TE morphology of blastocysts was scored according to the criteria described by Gardner and Schoolcraft (22) with minor differences as follows: grade A, TE with many cells forming a cohesive epithelium; grade B, TE with few cells forming a loose epithelium; grade C, TE with very few large cells or not hatched from zona pellucida.

Blastocyst Biopsy and Vitrification

On the morning of day 6, all blastocysts of the patient were biopsied simultaneously. Blastocysts were placed in a drop of G-MOPS (Vitrolife). The blastocyst was positioned using the holding pipette to locate the herniating TE at the 3 o'clock position. A piece of TE away from the inner cell mass was aspirated with a biopsy pipette (internal diameter, 30 μm) and dissected with a Zilos TK laser (Hamilton Thorne).

Biopsied TE cells were prepared for FISH, and the blastocysts were vitrified within 1–2 hours after TE biopsy using a Kitazato vitrification kit (Kitazato Biopharma) in combination with closed High Security Vitrification Straws (Cryo Bio System). The vitrification procedure was performed according to the manufacturer's protocols. Each blastocyst was stored in an individual straw.

FISH Analysis

Briefly, the biopsied TE piece was exposed for 5 minutes to hypotonic solution (1% sodium citrate in 6 mg/mL bovine serum albumin) and transferred into a small drop of Tween 20 fixative (0.01 N HCl, 0.1% Tween 20) on a clean slide. The TE piece suspended in the fixative drop was scattered mechanically using the capillary glass needle (internal diameter, 5 μm), and the TE cells were fully spread out on the slide. The FISH procedure was performed as we have described elsewhere (5). The designed probe set contained sufficient probes to detect all expected unbalanced forms of the chromosomal rearrangement according to the European Society for Human Reproduction and Embryology PGD consortium best practice guidelines for FISH-based PGD (23). For Robertsonian translocations and pericentric inversions, a set of two or three DNA probes were used, located on the chromosomes involved. For reciprocal translocations, a set of three or four DNA probes were used, flanking the breakpoints on the chromosomes involved. FISH analysis was also used for sex chromosomal aneuploidy, sex determination of X-linked disorders, and

Y-chromosome microdeletion by a set of three centromere DNA probes (CEP 18/X/Y).

Blastocyst Warming, Transfer, and Luteal Support

For the frozen ET (FET) cycle, no more than two blastocysts were transferred to each patient. According to the number of blastocysts to be warmed, we prioritized the blastocysts for warming based on the best quality before biopsy. Blastocysts were warmed using a commercially available warming solution (Kitazato Biopharma), according to the manufacturer's instructions. After warming, blastocysts were transferred to G2.5 medium and cultured for 2–6 hours. Only blastocysts that reexpanded after warming were considered as surviving and suitable for transfer.

The blastocysts were transferred either 5 days after ovulation in a natural cycle or 5 days after the initiation of P therapy with an endometrial preparation containing estradiol valerate and P. Briefly, 6 mg of estradiol valerate was administered from day 3 for 10–15 days, and luteal support was applied when satisfactory endometrial development (thickness $\geq$8 mm) was confirmed by ultrasound examination.

Outcome Measures and Statistical Analysis

Clinical pregnancy was defined as at least one intrauterine gestational sac with cardiac action by ultrasound performed 45 days after ET. Biochemical pregnancy was defined as positive hCG without any intrauterine gestational sac. Abortion was defined if the intrauterine gestational sac disappeared before 20 weeks of gestation. In this study, single ET cycles and double ET cycles with no or two gestational sacs were included for comparing the implantation outcome.

Categorical variables were presented as percentages and compared using χ^2 or Fisher's exact tests. The linear-by-linear association test was used to examine linear associations between the diagnosed failure rate, survival, or implantation outcomes and biopsied TE cell number. For continuous variables, maternal age was presented as mean $\pm$ SD, and the number of biopsied TE cells was presented as median (range). The numbers of biopsied TE cells were compared using the Kruskal-Wallis *H* test. A bivariate logistic regression model was used to assess the relationship among the embryologist, female age, TE morphological score, biopsied TE cell number, and clinical outcomes of FET cycles. $P<.05$ was considered statistically significant. Analyses were performed using the statistical package SPSS, version 19.0 (*SPSS*).

RESULTS

Clinical Outcome

A total of 3,097 blastocysts were biopsied in 638 cycles. After FISH analysis, 2,994 blastocysts had reliable results (96.7%), and the number of diagnosed normal blastocysts was 1,534 (51.2%). FET was performed for 480 biopsied cycles, and 317 cycles yielded a pregnancy (66.0%). The other 158 cycles were not transferred owing to no chromosomally normal blastocysts (103 cycles), no survived embryos (20 cycles), or deferred FET (35 cycles). A total of 909 blastocysts were warmed, 820 blastocysts survived (90.2%), and 395 blastocysts were implanted (48.2%; Supplemental Table 1).

Effect of TE Quality and Personnel Experience on biopsied TE Cell Number

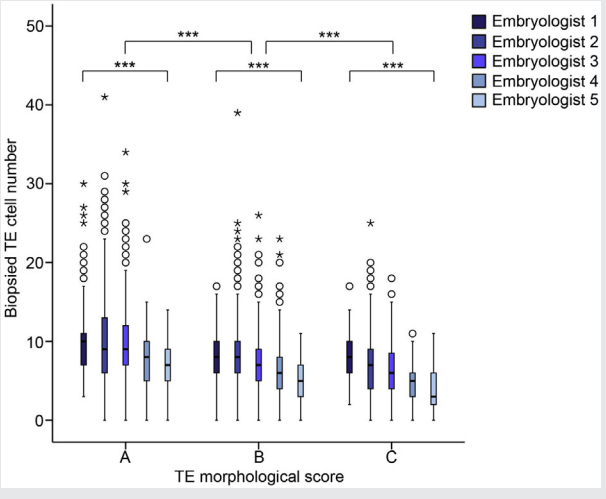
Among 3,097 biopsied blastocysts, the median number of biopsied TE cells was seven (range, 0–41). Classification of the TE quality revealed 889 grade A, 1,349 grade B, and 859 grade C. Blastocysts with better TE morphological scores showed a higher biopsied cell number ($P<.001$). The median number of biopsied TE cells was nine (range, 0–41), seven (range, 0–39), and six (range, 0–25) in the grade A, B, and C TE score subgroups, respectively.

TE biopsies were performed by five embryologists, with a total of 450, 1,104, 748, 627, and 168 blastocysts biopsied by the individual personnel, respectively. The median number of biopsied TE cells from each embryologist was nine (range, 0–30), eight (range, 0–41), eight (range, 0–34), six (range, 0–23), and 5.5 (range, 0–14), respectively. There were significant differences in the biopsied cell number among those obtained by the five embryologists ($P<.001$). Significant differences were observed in the median number of cells in the biopsies in the grade A, B, and C TE score subgroups obtained by the different embryologists (Fig. 1; Supplemental Table 2).

The Effect of Biopsied TE Cell Number on Diagnostic Efficiency

TE cell nuclei were not found after FISH analysis in 59 blastocysts (1.9%). When there was only one nucleus, 13.3% of the samples lacked diagnosis owing to no or obscure fluorescence signals. The diagnosed failure rates were 8.3%, 4.0%, 3.2%, and 1.3% when two, three, four, and five nuclei existed, respectively, and then became negligible if six (0.7%), seven (0.6%), eight (0.6%), nine (0.3%), or more (0%) nuclei were found (*P* trend $<$ .001; Fig. 2).

FIGURE 1



Median number of biopsied trophectoderm (TE) cells according to the different embryologists stratified by TE morphological score. *Extreme value of biopsied TE cell number; ***$P<.001$.

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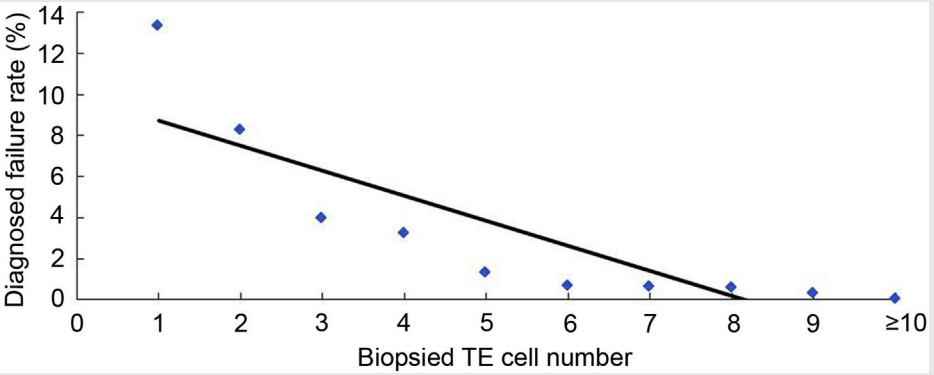
FIGURE 2



Diagnosed failure rates decline along with the increasing biopsied trophectoderm (TE) cell mumber. Continuous line shows trend line. *P* trend < .001.

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The Effect of Biopsied TE Cell Number on Clinical Efficiency

Since the embryologist, female age, and TE quality might be the confounders for the final clinical outcome, we first conducted a logistic regression analysis. The results showed that only TE morphological score was predictive of blastocyst survival capacity and that the TE morphological score and biopsied TE cell number were both influencing factors for blastocyst implantation potential (Supplemental Table 3).

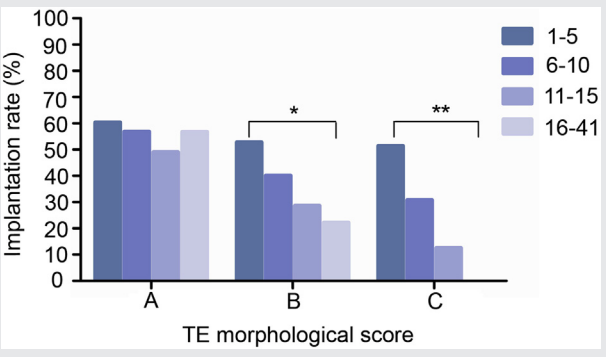
Blastocysts were divided into four groups arbitrarily according to the biopsied cell number: 1–5, 6–10, 11–15, and 16–41. To adjust for the influence of TE quality, blastocysts were stratified by TE morphological score for investigating the correlation between biopsied TE cell number and clinical outcomes. In all three subgroups with different TE morphological scores, no differences were observed in the survival rates among the four biopsied TE cell number groups ($P>.05$, P trend $> .05$). In the grade A TE score subgroup, the implantation rates were 60.4%, 56.9%, 49.0%, and 56.8% in the 1–5, 6–10, 11–15, and 16–41 biopsied TE cell number groups, respectively ($P=.695$, P trend $= .49$). In the grade B TE score subgroup, the implantation rates were 52.8%, 40.1%, 28.6%, and 22.2% in the 1–5, 6–10, 11–15, and 16–41 biopsied TE cell number groups, respectively ($P=.135$, P trend $= .019$). In the grade C TE score subgroup, the implantation rates were 51.4%, 30.8%, 12.5%, and 0% in the 1–5, 6–10, 11–15, and 16–41 biopsied TE cell number groups, respectively ($P=.063$, P trend $= .008$; Fig. 3; Supplemental Table 4).

In all three subgroups of different TE scores, the biochemical loss rates and abortion rates both had no differences in the 1–5, 6–10, 11–15, and 16–41 biopsied TE cell number groups ($P>.05$; Supplemental Table 4). Five couples chose to detect the chromosomal status of the chorionic villi from aborted embryos using comparative genomic hybridization (CGH), and all five embryos were chromosomal normal.

DISCUSSION

In this retrospective study, the FISH analysis enables a better assessment of the number of TE cells sampled for PGD. The median number of biopsied TE cells was higher in the blastocysts with higher TE morphological scores, reflecting the positive correlation between the biopsied cell number and TE quality. It has been shown that the density of TE cells increases in blastocysts with better TE quality (24). Therefore, for the same size, the biopsied cell number should be higher in the blastocysts with better TE quality. The personnel experience of different embryologists is also an influencing factor. Unlike the cleavage-stage embryo biopsy, the number of biopsied cells in the blastocyst biopsy is hard to quantify and largely dependent on the experience of embryologist. The fact that significant differences in the median number of biopsied cells exist among different embryologists indicates that a detailed biopsy training standard is needed when shifting from cleavage biopsy to blastocyst biopsy.

FIGURE 3



Implantation rate according to the different biopsied trophectoderm (TE) cell number stratified by TE morphological score. **P* trend < .05; ***P* trend < .01.

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In cleavage-stage embryo biopsy cycles, the diagnosed failure rate after FISH analysis was 12.8% when one cell was biopsied in our center (5), and some studies showed a small benefit of two-cell over one-cell biopsy on diagnostic efficiency (25, 26). In this study performed with blastocyst biopsy, when one cell was biopsied, the diagnosed failure rate (13.3%) was comparable to that of the cleavage-stage embryo biopsy, then decreased with more biopsied TE cells, suggesting that the diagnostic efficiency of FISH increased with more analyzed cells. In our experience, if some nuclei were damaged (debris), or the fluorescence signals were doubtful (split spot), additional nuclei could help to achieve a result.

Recently, several studies have indicated that TE morphology, rather than inner cell mass morphology and blastocoel expansion, is the most important parameter for predicting pregnancy outcome in fresh ET cycles (27) or in FET cycles (28). Stratification by TE morphological score allowed comparison of the clinical efficiency of different biopsied TE cell number groups on a relatively uniform TE quality level. We found no negative effects of the higher biopsied TE cell number on embryo survival rate, regardless of the TE quality. In this study, blastocyst survival was defined as reexpansion after warming, a process that requires the TE cells to pump water into the blastocoel (29). It is unclear how many TE cells are required for blastocyst expansion, but studies of the blastocyst developmental potential from single blastomeres (30, 31) indicate that only a few cells are required for blastocoel formation, thus partly explaining why the blastocyst survival capacity was not affected by the removal of more cells.

The contribution of TE for successful implantation obviously depends on a certain number of functional TE cells. After TE biopsy, the implantation potential of the blastocyst may be affected by the cell loss. The fact that the implantation rates had a gradually decreasing tendency with increasing biopsied cell number only in the grades B and C TE score subgroups indicates that the TE quality of blastocysts correlates directly with tolerance to cell loss. For the blastocysts with a grade A TE score, the implantation rate did not decline even when 16–41 TE cells were removed, but this did not mean that embryologists could perform TE biopsies of an unlimited number of cells because the number of biopsied cells in this study might not reach the critical value. In the grade B and C TE score subgroups, when 6–10 cells were biopsied, the implantation rate of blastocysts decreased compared with that achieved when 1–5 cells were biopsied, although this effect did not reach the level of statistical significance, possibly owing to the small sample size. It is suggested that, for blastocysts with grade B or C TE scores, 1–5 cells may be the appropriate biopsied TE cell number to maintain the implantation potential. Although fewer cells do not favor the diagnostic efficiency, it seems not to be a problem considering the high efficiency of reexamining the test-failure embryos in PGD cycles (32).

The CGH results of the aborted embryos suggested that aneuploidy was not involved in miscarriage, so the interaction between embryo and endometrium, such as TE secretion of hCG, might be the main reason for abortion. Although the sample size was small, our data showed that the biopsied TE cell number was unrelated to embryo biochemical loss and abortion. A large-scale study was needed to determine whether the biopsied cell number would affect embryonic development after implantation.

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SUPPLEMENTAL TABLE 1

Baseline characteristic of patients and clinical outcomes of PGD cycles.

Characteristic	Robertsonian translocations	Reciprocal translocation	Inversion	Sex chromosome aneuploidy	X-linked genetic disease	Y chromosome microdeletion	Total
No. of patients	158	315	65	31	2	18	589
Biopsied cycles	173	347	67	31	2	18	638
Maternal age, mean ± SD	29.12 ± 3.26	28.51 ± 3.29	29.21 ± 3.65	29.06 ± 3.73	35.0 ± 2.83	27.17 ± 3.11	28.76 ± 3.37
Biopsied embryos	826	1655	383	152	4	77	3097
Diagnosed embryos (%)	807 (97.7)	1589 (96.0)	369 (96.3)	150 (98.7)	4 (100.0)	75 (97.4)	2994 (96.7)
Transferable embryos (%)	490 (60.7)	592 (37.3)	300 (81.3)	117 (78.0)	2 (50.0)	33 (44.0)	1534 (51.2)
Warmed embryos	301	404	128	47	2	27	909
Survived embryos[a] (%)	270 (89.7)	365 (90.3)	116 (90.6)	43 (91.5)	2 (100.0)	24 (88.9)	820 (90.2)
Transferred cycles	145	231	60	25	2	17	480
Cumulative clinical pregnancy[b] (%)	100 (69.0)	142 (61.5)	49 (81.7)	17 (68.0)	1 (50.0)	8 (47.1)	317 (66.0)
Embryos implanted (%)	133 (49.3)	168 (45.8)	63 (54.3)	21 (48.8)	1 (50.0)	9 (37.5)	395 (48.2)
Miscarriage (%)	8 (8.0)	15 (10.6)	3 (6.1)	4 (23.5)	0	0	30 (9.5)

[a] All survived embryos were transferred to patients.
[b] The cumulative clinical pregnancy was used because in some PGD cycles, several FET cycles were performed after one biopsied cycle.

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SUPPLEMENTAL TABLE 2

The effect of personnel experience on the biopsied cell number.

Variable	Manipulator 1	Manipulator 2	Manipulator 3	Manipulator 4	Manipulator 5	*P*[a] value
Grade A TE score						
No. of embryos	170	322	227	107	63	
Biopsied cell number, median (range)	10 (3–30)	9 (0–41)	9 (0–34)	8 (0–23)	7 (0–14)	< .001
Grade B TE score						
No. of embryos	165	357	381	384	62	
Biopsied cell number, median (range)	8 (0–17)	8 (0–39)	7 (0–26)	6 (0–23)	5 (0–11)	< .001
Grade C TE score						
No. of embryos	115	425	140	136	43	
Biopsied cell number, median (range)	8 (2–17)	7 (0–25)	6 (0–18)	5 (0–11)	3 (0–11)	< .001
Total						
No. of embryos	450	1104	748	627	168	
Biopsied cell number, median (range)	9 (0–30)	8 (0–41)	8 (0–34)	6 (0–23)	5.5 (0–14)	< .001

[a] *P* values for testing overall differences between the groups.

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SUPPLEMENTAL TABLE 3

Logistic regression analysis for embryo survival and implantation.

Variable	*P*	Odds ratio	LCI	UCI
Embryo survival				
TE = A		1		
TE = B	.018	0.499	0.281	0.886
TE = C	.000	0.174	0.097	0.311
Embryo implantation				
Biopsied cell nos. 1–5		1		
Biopsied cell nos. 6–10	.025	0.625	0.414	0.943
Biopsied cell nos. 11–15	.003	0.414	0.231	0.744
Biopsied cell no. ≥16	.062	0.519	0.261	1.033
TE = A		1		
TE = B	<.001	0.503	0.348	0.726
TE = C	<.001	0.375	0.229	0.615

Note: LCI = lower confidence interval; TE = trophectoderm; UCI = upper confidence interval.

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SUPPLEMENTAL TABLE 4

Survival and implantation outcome according to the different biopsied cell number stratified by TE morphological score

Variable	Grade A TE score				Grade B TE score				Grade C TE score			
Biopsied TE cell number	**1–5**	**6–10**	**11–15**	**>15**	**1–5**	**6–10**	**11–15**	**>15**	**1–5**	**6–10**	**11–15**	**>15**
No. of embryos	161	444	172	101	399	728	143	47	349	403	77	14
Diagnosed embryos	159	444	172	101	382	727	143	47	331	397	77	14
Warmed embryos	70	202	73	59	82	210	36	21	55	83	15	3
Survived embryos	67	193	68	56	72	194	31	19	44	62	11	3
Survival rate, %	95.7	95.5	93.2	94.9	87.8	92.4	86.1	90.5	80.0	74.7	73.3	100.0
Transferred embryos	67	193	68	56	72	194	31	19	44	62	11	3
Embryos with definite implantation outcome[a]	48	153	51	37	53	137	21	9	35	52	8	2
Embryos with negative hCG	15	50	19	14	18	51	14	6	13	32	6	2
Embryos with positive hCG												
Undefinite biochemical pregnancy loss[b]	4	4	3	1	2	19	1	1	3	3	1	0
Definite biochemical pregnancy loss[c]	0	12	4	1	5	12	0	0	1	1	0	0
Biochemical loss rate (%)[d]	0	12.1	13.8	4.5	15.2	17.9	0	0	5.3	5.9	0	0
Implanted	29	87	25	21	28	55	6	2	18	16	1	0
Implantation rate (%)	60.4	56.9	49.0	56.8	52.8[f]	40.1[f]	28.6[f]	22.2[f]	51.4[g]	30.8[g]	12.5[g]	0[g]
Embryos with definite abortion outcome[e]	25	73	23	19	20	43	5	2	16	13	1	
Embryos aborted	3	6	1	1	2	5	0	0	0	1	0	
Abortion rate, %	12.0	8.2	4.3	5.3	10.0	11.6	0	0	0	7.7	0	

[a] Embryos with definite implantation outcomes were from single ET cycles or double ET cycles with no or two gestational sacs.
[b] Embryos with indefinite biochemical pregnancy loss were from double ET cycles for which the origin of elevated serum hCG was not certain.
[c] Embryos with definite biochemical pregnancy loss were from single ET cycles.
[d] Biochemical loss rate was defined as (No. of embryos with definite biochemical loss)/(No. of embryos with definite biochemical loss + No. of embryos implanted).
[e] Embryos with definite abortion outcome indicated that all implanted embryos were aborted or not aborted.
[f] *P* trend = .019.
[g] *P* trend = .008.

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