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Original Article

Detection of early cleavage embryos improves pregnancy and delivery rates of Day 3 embryo transfer during *in vitro* fertilization

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ABSTRACT

Objective: This study established a simple criterion for improving the pregnancy and delivery rates of Day 3 embryo transfer for *in vitro* fertilization (IVF) by assessing the early cleavage of two-cell stage embryos.

Materials and Methods: In total, 258 cycle patients undergoing an IVF and Day 3 embryo transfer program were recruited. All cycles were divided into four groups containing viable Day 3 embryos and those (A) with distinct early cleavage (equal-sized blastomeres and ≤10% fragmentation: ECA grade); (B) with indistinct early cleavage (equal sized blastomeres, >2 blastomeres, or >10% fragmentation: ECB grade); (C) without early cleavage [no early cleavage (NEC grade)]; or (D) without early cleavage being assessed (control) at 25–27 after insemination.

Results: The percentage of viable Day 3 embryos from ECA grade (75.1%, 507/675) was significantly higher than that from ECB grade (19.2%, 151/403) or NEC grade (27.1%, 127/469) embryos ($p < 0.01$). The pregnancy and delivery rates in the ECA group [65.7% (65/990) and 48.5% (48/990), respectively] were significantly higher than those in the ECB group [30.8% (4/13) and 7.7% (1/13), respectively] or NEC group [36.8% (14/38) and 23.7% (9/38), respectively; all $p < 0.01$]. The implantation rate in the ECA group (32.3%, 129/400) was higher than those in the ECB (6.8%, 4/59) and NEC (13.0%, 18/136) groups ($p < 0.01$).

Conclusion: Simple selection using the early cleavage morphology may improve the pregnancy and delivery rates of Day 3 embryo transfer programs.

Introduction

A simple and efficient method for increasing pregnancy and implantation rates to a high level by using advance predictors of the transferred embryo quality is crucial for those involved in assisted reproductive technology [1–3]. A novel indicator, early cleavage at the two-cell stage, has been suggested for assessing human pre-implantation embryonic quality during *in vitro* fertilization (IVF) [4,5]. The earliest zygotic division occurs 20–27 hours after insemination or intracytoplasmic sperm injection (ICSI) [5–7]; hence, the recommended time for observing early cleavage is 25–27 hours [4,8]. At 24 and 27 hours after insemination, 5% and 38% of fertilized zygotes demonstrate early cleavage, respectively [6,9]. Early cleavage embryos have a higher blastocyst formation rate, superior morphology, and higher implantation rate compared with embryos without early cleavage [4,10,11]. However, the relationship between the morphological characteristics of early cleavage at the two-cell stage and embryo quality prior to the transfer warrants further discussion. Although the assessment of the degree of fragmentation and number and size of blastomeres is useful in determining the embryo quality prior to the transfer on Day 3, the usefulness of such assessment for determining the outcomes of

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viable Day 3 early cleavage embryos with different morphologies remain unknown. The assessment of the Day 3 embryo quality and early cleavage is a useful criterion for evaluating Day 3 embryo transfers. For selecting transferrable embryos, the morphological characteristics of early cleavage embryos may be as crucial as those of Day 2, 3, 4, or 5 embryos. For example, the degree of fragmentation is a major indicator of embryo quality on Days 2–5 prior to the transfer. Furthermore, certain fragmentation patterns occur during the one- or two-cell stage, resulting in a loss of certain standard proteins from the blastomeres [12], which are associated with apoptosis [13]. Thus, the assessment of the morphological characteristics of early cleavage at the two-cell stage should be a criterion for embryo quality prediction.

A single evaluation of cell number and morphology on Day 3 of culture is not correlated with pregnancy rate or blastocyst formation [7,8]. The assessments of early cleavage appearance, Day 2 and 3 embryo morphology, or irregular development are primary indicators for selecting embryos for transfer on Day 3; however, the selection protocol should be efficiently shortened and simplified to reduce the range of efficient viable Day 3 embryos selected for transfer. In this study, two factors—morphology of early cleavage and quality of Day 3 embryos—were assessed prior to the transfer. This study established a simple and efficient selection criterion for Day 3 embryo transfer and predicted the optimal embryo quality and outcomes after the transfer.

Materials and methods

Patients and oocyte retrieval

We analyzed a database containing the clinical and laboratory information of all IVF treatment cycles conducted at the Lee Women's Hospital between September and December 2009. The study protocol was approved by the Institutional Review Board of Chung Shan Medical University Hospital (CS10084). The ovarian stimulation and embryo culture procedures used in our IVF program have been published elsewhere [14]. The participating women were administered the gonadotropin-releasing hormone (GnRH) agonist leuprolide acetate (Lupron, Takeda Chemical Industries, Ltd., Osaka, Japan) from the midluteal phase for pituitary downregulation. A serum estradiol (E_2) level of <50 pg/mL on cycle Day 2 confirmed pituitary suppression, and recombinant follicle-stimulating hormone (Gonal-F, Serono) treatment was initiated. The participants' ovarian responses were monitored through serial serum E_2 levels and ultrasound examinations. When the leading follicles reached approximately 18 mm in diameter with an appropriate serum E_2 level, 10,000 IU of human chorionic gonadotrophin (Profasi, Serono) was administered. Transvaginal oocyte retrieval was performed 34–36 hours later.

Embryo fertilization and culture

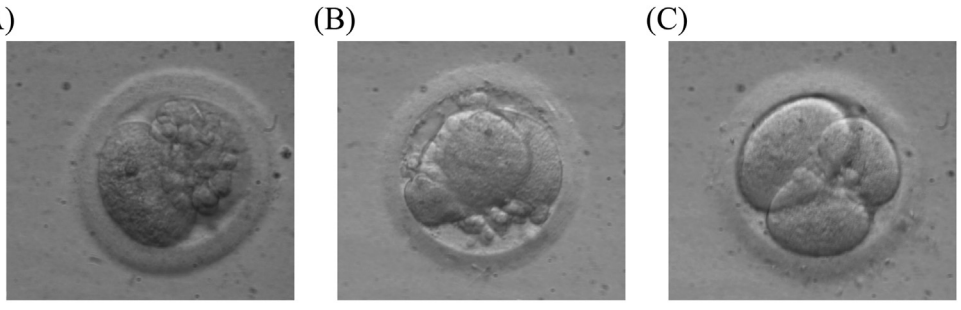
All mature oocytes were used for artificial insemination or ICSI. All inseminations and IVF were performed using microdrops of human tubal fluid medium (mHTF; Irvine Scientific, Santa Ana, CA, USA) containing a 5% (v/v) serum substitute supplement (Irvine Scientific). Immediately prior to ICSI, cumulus cells were removed by pipetting the oocytes in mHTF containing 80 IU/mL hyaluronidase (Type 8, H-3757; Sigma Chemical, St. Louis, MO, USA). Following artificial insemination or ICSI, all embryos were further cultured in microdrops of Quinns Advantage Cleavage (SAGE) medium.

Criteria for early cleavage and embryonic development

After insemination, embryonic development—including the embryonic pronuclei appearance (18–20 hours), two-cell stage or early cleavage (26–27 hours), four-cell stage (45–46 hours), and eight-cell stage (69–70 hours)—was observed. On the basis of the early cleavage morphology, two-cell stage embryos were classified as follows: (1) ECA grade, an embryo with two equal-sized blastomeres and ≤10% fragmentation; (2) ECB grade, an embryo with two unequal-sized blastomeres, more than two blastomeres, or >10% fragmentation; and (3) no early cleavage (NEC) grade; embryos without division at the time for assessment were defined as NEC. In the ECB grade, all embryos were further divided into ECP (>10% fragmentation), EC > 2 (>2 blastomeres), and ECC (unequal sized blastomeres) grades according to their two-cell stage morphology (Figure 1). Day 3 embryos with ≥8 equal-sized blastomeres and ≤20% fragmentation were considered advanced or viable embryos. By contrast, the embryos with <8 equal- or unequal-sized blastomeres or >20% fragmentation were considered poor embryos.

Assessment of the relationship between Day 3 embryo quality and early cleavage morphology at the two-cell stage

On the basis of their embryo development protocols, the 258 IVF cycle patients were classified into two groups: (1) the control



	ECP	ECC	EC > 2
Day 3 group			
Total embryos	150	165	88
No. of good embryos on Day 3 [%]	15 [10.0]	83 [50.3[a]]	53 [60.2[a]]

Figure 1. The two-cell stage morphology and distribution of early cleavage embryos with >10% fragmentation (A), unequal-sized blastomeres (B), and >2 blastomeres (C). All of them were included in the ECB group. ECB = early cleavage grade B (unequal sized blastomeres, >2 blastomeres, or >10% fragmentation); ECP = early cleavage with poor quality (>10% fragmentation).

group, which included 30 patients with no observation of early cleavage at the two-cell stage, and (2) the early cleavage assessment group, which included 228 patients with early cleavage assessment. Both groups underwent the Day 3 transfer protocol.

To assess the relationship between the early cleavage morphology and embryo quality on Day 3, viable embryos graded on the basis of the early cleavage morphology were evaluated and compared. In this step, the quality of the early cleavage assessment group embryos was analyzed.

Comparison of pregnancy rates between groups classified according to the early cleavage morphology

Only the viable Day 3 embryos classified according to the early cleavage morphology were selected for transfer. Patients receiving at least two viable ECA-grade embryos were categorized into the ECA group. Patients receiving at least two viable ECB-grade embryos but to whom <2 viable ECA-grade embryos were transferred were categorized into the ECB group. Patients receiving at least two viable NEC-grade embryos but to whom <2 viable ECA- or ECB-grade embryos were transferred were categorized into the NEC group. The differences in pregnancy and implantation rates were compared among these groups. However, only 150 of the 228 early cleavage assessment group cycles were corrected after this transfer principle was used, whereas the remaining 78 cycle patients that were processed were using zygote cryopreservation, blastocyst cryopreservation, <2 viable embryo transfer, or no embryo transfer.

Statistical analysis

The embryos with or without early cleavage on Day 3 were compared through a Chi-square test analysis. A confidence level of $p < 0.05$ was considered statistically significant.

Results

ECA group exhibited a high percentage of good Day 3 embryos

We retrieved 1779 oocytes from 228 patients who underwent IVF and assessments of the relationship between the early cleavage morphology and Day 3 embryo quality (Table 1). From the retrieved oocytes, 1697 mature oocytes underwent artificial insemination or ICSI, and 1547 (91.2%) embryos displayed cleavage. At the two-cell stage, the proportion of early cleavage embryos was 69.7% (1078/1547), of which 62.6% (675/1078) and 37.4% (403/1078) were ECA and ECB embryos, respectively. The total percentage of viable Day 3 embryos was 50.7% (785/1547); of these, 658 (85.1%, 658/785) embryos showed early cleavage at the two-cell stage, and 507 (77.1%) and 151 (22.9%) were ECA- and ECB-grade embryos, respectively. The percentage of viable Day 3 embryos from ECA-grade (75.1%, 507/675) was significantly higher than that of viable Day 3 embryos from ECB-grade (37.5%, 151/403) or NEC-grade embryos (27.1%, 127/469, $p < 0.01$). The percentage of viable Day 3 embryos from NEC-grade embryos was significantly higher than that of viable Day 3 embryos from ECB-grade ($p < 0.01$).

Table 1
Early cleavage morphology and embryo quality.

	Day 3 transfer
No. of cycles	228
Female age (y)	33.4 ± 0.02
No. of oocytes retrieved	1779
Mature oocytes (MII)	1697
No. of embryos with normal fertilization (%, per total MII)	1547 (91.2)
No. of early cleavage (%, per total cleavage embryos)	1078 (69.7)
No. of Day 3 good embryos (%, per total embryos)	785 (50.7)
Day 3 good embryos with early cleavage	658 (83.8)
Assessment of embryo quality	
No. of ECA at 2 cell stage (%, per total early cleavage)	675 (62.6)
No. of ECB at 2 cell stage (%, per total early cleavage)	403 (37.4)
No. of Day 3 good embryos derived from ECA (%, per total ECA)	507 (75.1)
No. of Day 3 good embryos derived from ECB (%, per total ECB)	151 (19.2[a])
No. of Day 3 good embryos derived from NEC (%, per total NEC)	127 (27.1[a,b])

ECA = early cleavage grade A (equal-sized blastomeres and <10% fragmentation); ECB = early cleavage grade B (unequal sized blastomeres, >2 blastomeres, or >10% fragmentation); NEC = no early cleavage.
[a] Compared with ECA, $p < 0.01$.
[b] Compared with ECB, $p < 0.01$.

ECA-grade embryos exhibited higher pregnancy rates

Embryo transfer was performed in 209 of 228 patients; however, 59 of these patients who received only one poor embryo or to whom both/all transferred embryos are not the same early cleavage grade were excluded. Finally, 595 embryos were transferred into 150 patients, resulting in 83 pregnancies (55.3%). According to the morphological characteristics of the two-cell stage for the transferred embryos, all patients were classified into the ECA ($n = 99$), ECB ($n = 13$), and NEC ($n = 38$) groups (Table 2). The early cleavage groups (ECA and ECB) displayed significantly higher pregnancy (61.6%, 69/112) and delivery (43.8%, 49/112) rates than the NEC group (pregnancy rate: 36.8%, 14/38; delivery rate: 23.7%, 9/38) did (both $p < 0.05$); this result was primarily attributed to the ECA group. The pregnancy (65.7%, 65/99), implantation (32.3%, 129/400), and delivery (48.5%, 48/99) rates of the ECA group were significantly higher than those of the ECB (pregnancy rate: 30.8%, 4/13; implantation rate: 6.8%, 4/59; delivery rate: 11.1%, 1/13) and NEC (pregnancy rate: 36.8%, 14/38; implantation rate: 13.0%, 18/136; delivery rate: 23.7%, 9/38) groups (all $p < 0.01$). The rates did not differ significantly between the ECB and NEC groups. The results indicated that among the early cleavage groups, ECA generated the most satisfactory pregnancy rate. Furthermore, the control group, without two-cell stage morphology information, exhibited results similar to those of the ECA group, indicating that selecting viable Day 3 embryos could result in favorable outcomes, even without observation of the two-cell stage; nevertheless, early cleavage morphology can aid in selecting and distinguishing between superior and inferior embryos for Day 3 transfer. Thus, not all viable Day 3 embryos are ideal for transfer.

Early cleavage embryos with >10% fragmentation exhibited the lowest quality on Day 3

The morphologies of ECP-, ECC-, and EC > 2-grade embryos are provided in Figure 1; their distribution on Day 3 was 150 (37.2%), 165 (39.3%), and 88 (21.8%), respectively. Furthermore, 10.0% (15/150) of the ECP-grade embryos developed into viable Day 3 embryos, which was significantly lower than the number of viable Day 3 embryos from ECC-grade (50.3%, 83/165) and EC > 2-grade (60.2%, 53/88).

Discussion

We assessed the results of applying different embryo selection criteria and observed that the ECA-grade embryos, with distinct early cleavage, showed superior development and higher pregnancy and implantation rates. In this study, 25–27 hours after artificial insemination or ICSI, 69.7% (1078/1547) of the total embryos displayed early cleavage, and 61.0% (658/1078) of them developed into viable Day 3 embryos (Table 1). These findings

Table 2
Comparison of the pregnancy rates among different groups classified on the basis of the early cleavage morphology.

Group	ECA	ECB	NEC	No observation of early cleavage
Transferred cycles	99	13	38	30
Age (y)	32.9 ± 0.21	35.2 ± 0.54	35.9 ± 0.12	32.4 ± 0.13
No. of total mixed transferred embryos	400	49	136	118
No. of pregnancies (%, per embryo transfers)	65 (65.7)	4 (30.8[a,b])	14 (36.8[a,b])	19 (63.3)
No. of sac (%, per embryos transferred)	129 (32.3)	4 (8.2[a])	18 (13[a])	24 (20.3)
No. of miscarriage (%, per pregnancies)	14 (21.5)	1 (25.0)	4 (28.6)	5 (27.8)
No. of ectopic	1	0	1	1
No. of IUFD	2	2	0	1
No. of deliveries (%, per embryo transfers)	48 (48.5)	1 (7.7[a])	9 (23.7[a])	12 (40.0)
No. of singletons (%, per deliveries)	26 (54.2)	1 (100)	8 (88.9)	10 (83.3)
No. of twins (%, per deliveries)	22 (45.8)	0 (0)	1 (11.1)	2 (20.0)

ECA = early cleavage grade A (equal-sized blastomeres and <10% fragmentation); ECB = early cleavage grade B (unequal sized blastomeres, >2 blastomeres, or >10% fragmentation); IUFD = intrauterine fetal demise. NEC = no early cleavage.
[a] Compared with ECA group, $p < 0.01$.
[b] Compared with no observation of early cleavage group, $p < 0.05$.

correlate well with those of previous studies, suggesting that early cleavage is an indicator of higher human embryo quality [4,5,10,11,15]. The results of this study further indicated that a few factors affect the efficiency of predicting Day 3 embryo quality with regard to early cleavage morphology. First, the embryos with early cleavage (ECA and ECB groups) exhibited a higher potential to develop into viable Day 3 embryos (61.0%, 658/1078; Table 1); thus, the appearance of early cleavage was an indicator of embryonic development potential. Second, the early cleavage morphology, including the fragmentation degree as well as blastomere number and size at the two-cell stage, excessively affected embryonic development. Depending on the early cleavage morphology, all embryos could be divided into ECA and ECB groups, with most being classified into the ECA group (77.1%, 507/658). Furthermore, of all 785 included embryos, most viable Day 3 embryos were from ECA grade (64.6%, 507/785), indicating that most early cleavage embryos exhibit two equal-sized blastomeres with ≤10% fragmentation and that they have a higher potential to develop into viable Day 3 embryos. Thus, we suggest that ECA-grade embryos have a higher potential to develop into viable Day 3 embryos than those with unequal sized or >2 blastomeres or with >10% fragmentation (ECB) do. Although the ECB group can further be divided into different subgrades, including ECP (>10% fragmentation), EC > 2 (>2 blastomeres), and ECC (unequal sized blastomeres), the number of embryos with these subgrades was small, and we could not compare the results; nevertheless, the irregular early cleavage morphology significantly affected the percentages of viable Day 3 embryos. The percentage of viable Day 3 embryos with >10% fragmentation was significantly lower in the ECB group, indicating that more fragmentation at the two-cell stage may generate the least Day 3 embryonic development. Although the fragmentation of human embryos commonly occurs in *in vitro* culture systems, with approximately 7% of the pronuclei fragmenting at the one-cell stage, certain fragmentation patterns occurring during the one- or two-cell stage result in the loss of certain standard apoptosis-associated proteins from the blastomeres [12,13]. The apoptotic and necrotic processes [13] can adversely affect embryonic development [16]; nevertheless, we suggest that these processes occur after fragmentation at the two-cell stage and continually reduce the quality of the developing embryo.

The percentage of viable Day 3 ECA-grade embryos was higher than that of viable Day 3 ECC-grade embryos (Figure 1). Embryos with unequal-sized blastomeres have higher multinuclearity rates and lower implantation rates [17], which are affected by the degree of fragmentation and multinuclearity [18]. Thus, we suggest that fragmentation and unequal size of blastomeres are the ideal markers for tracking embryonic development.

Here, irregular or rapid early cleavage (EC > 2) resulted in low embryonic development. At the Day 3 stage, the relationship between cell number and embryo quality differed from that at the two-cell stage. However, the embryos that cleaved very rapidly exhibited decreased developmental ability compared with those that cleaved at an appropriate speed [5,6]. The blastomere number in the patients who became pregnant after embryo transfer on Days 2 and 3 was higher than that in those who did not become pregnant [19]. However, dysmorphic and slow-developing or arrested embryos exhibit significantly more polyploidy and mosaicism than normally developing human embryos do [20]. Moreover, the percentage of viable Day 3 embryos in the NEC group was significantly higher than that in the ECB group, further suggesting that significant fragmentation or irregular cell size or division (>2 blastomeres), particularly with severe fragmentation at the two-cell stage, is a more effective indicator of inviable embryos than delayed first cleavage is. Hence, for the two-cell or Day 3 stage, combining the assessment of morphological characteristics and regular development is most critical in determining the embryo quality.

The results of the comparison of pregnancy rates among the groups were consistent with the previous results for predicting embryo quality at the two-cell stage. In addition, the subsequent embryonic development correlated well with the pregnancy rates [21,22]. Furthermore, the pregnancy rate was reported to be up to 46% when at least one embryo with early cleavage was transferred, and this success rate was significantly higher than that after embryos without early cleavage were used [15]. In the present study, the selection of viable embryos for transfer was based on the morphological characteristics on Day 3 of embryonic development. The pregnancy rate of the ECA group was highest among all groups. The pregnancy rate in the ECB and NEC groups was significantly lower than that in the ECA group and simply on Day 3 morphology groups. In the present study, the transfer of ECA-grade embryos on Day 3 also resulted in a higher delivery rate compared with that of ECB- and NEC-grade embryos. By contrast, the pregnancy rates after the Day 3 transfer of embryos with blindness to their early cleavage and after that of the ECB- and NEC-grade embryos did not differ significantly.

To increase successful implantation rates and reduce the potential for multiple pregnancies, we indicate that high-quality, early cleavage should be assessed before efficient viable embryos and blastocysts are selected for transfer. Because blastocyst formation may be influenced by long-term culture, age, air, and other conditions, for patients with fewer oocytes or embryos, emphasis should be placed on transferring viable Day 3 embryos with distinct early cleavage exhibiting two equal-sized blastomeres and ≤10% fragmentation, rather than on transferring viable embryos with

indistinct early cleavage; this maximizes the probability of a pregnancy. Patients with more retrieved oocytes should consider embryo transfer on Day 5 with expanding blastocysts showing distinct early cleavage; the remaining embryos can be cryopreserved at the blastocyst stage for a future cycle [14]. Regardless of the transfer protocol used, the early cleavage morphology is a crucial indicator of embryonic development and successful pregnancies as a result of human IVF.

In conclusion, Day 3 embryo quality is an effective predictor of a successful pregnancy; however, early cleavage is another crucial predictor of embryo transfer outcomes. A combination of these indicators could be a new criterion for selecting embryos for transfer and consequently increasing successful pregnancy rates, excluding inviable embryos, and reducing the number of embryos transferred, without reducing the pregnancy or delivery rates.

Conflicts of interest

The authors declare that they have no conflicts of interest relevant to this article.

Authors' contributions

C.I. Lee, T.H. Lee, and M.S. Lee contributed to conception and design. C.I. Lee, C.C. Huang, H.H. Chen, and C.H. Liu participated in data acquisition, analyses, and interpretation. C.I. Lee and H.H. Chen drafted the manuscript. T.H. Lee and M.S. Lee revised the manuscript for critical intellectual content. All authors read and approved the final version of the manuscript.

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