Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system

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Objective: To investigate the prevalence and potential causes of reverse cleavage (RC) by human early-cleavage embryos and its associations with embryonic development and implantation after transfer.
Design: Clinical retrospective cohort study.
Setting: Private fertility treatment center.
Patient(s): A total of 126 consecutive in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatment cycles, with 353 IVF and 436 ICSI embryos cultured in the Embryoscope until day 3.
Intervention(s): None.
Main Outcome Measure(s): Embryo assessment on day 3, incidence of abnormal division, embryo morphokinetic parameters, and fetal heart beat.
Result(s): RC, referring to either blastomere fusion or failed cytokinesis, occurred up to three times per individual embryo in 27.4% of embryos during the first three cleavage cycles. A higher incidence was associated with GnRH antagonist cycles compared with agonist cycles (odds ratio [OR] 1.683), or with ICSI compared with IVF (OR 1.600). After ICSI, sperm progressive motility was associated with RC (area under the receiver operating characteristic curve: 0.573). Compared with RC-negative embryos, a lower proportion of RC-positive embryos reached 6-cell stage or beyond by day 3 (47.7% vs. 71.7%), and were more likely to have multinucleation at the 4-cell stage (10.1% vs. 5.0%). Embryos showing RC had significantly poorer performance in both conventional grading and morphokinetic parameters, and they implanted less (0/22 vs. 29/131) than those not showing RC.
Conclusion(s): RC significantly compromised embryo development, culminating in poor implantation potential. For each embryo, it can occur on more than one occasion at any stage during the first 3 days of culture. It is associated with factors affecting both oocyte and sperm. (Fertil Steril® 2014;102:1295–300. ©2014 by American Society for Reproductive Medicine.)
Key Words: Reverse cleavage, abnormal cleavage pattern, time lapse, embryo development, implantation

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Embryo selection is a pivotal component of in vitro fertilization (IVF) programs, with morphology-based grading systems having been widely used for decades (1–4). As elective single-embryo transfer (eSET) gains increasing popularity to minimize multiple pregnancies following IVF (5), more dynamic approaches are required to identify for transfer the embryos with the highest implantation potential (6). One strategy to achieve this is to extend the culture period and see which embryos reach the developmental milestone of a blastocyst (7). However, despite the apparent health of babies born after

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the transfer of blastocysts (8, 9), concerns exist about the obstetrical, perinatal, and later outcomes of offspring conceived from embryos grown in an extended in vitro culture system (10–13), resulting in a call for the improved identification of early-cleavage embryos destined to become blastocysts (12). Time-lapse videography has been proposed for doing this (14).

Evidence has recently been reported with different time-lapse equipment or devices that indicates an improved prediction of implantation potential by analyzing the morphokinetics of human embryos at early cleavage stages (14–21). Time-lapse data help the embryologists to detect subtle timing differences between developmental milestones in embryos that appear to look the same (14, 16, 18–21). Recent publications have demonstrated compromised developmental and implantation potential of human embryos with a number of anomalous developments that are not apparent in conventional culture, including abnormal syngamy, first cytokinesis, cleavage patterns (22, 23), and blastomere fusion, termed reverse cleavage (RC) (24, 25). Such information is of practical value because higher priority for transfer can be given to embryos not showing such anomalies.

The present study investigated the occurrence and relevance of RC, including both blastomere fusion and failed cytokinesis which we have termed type I and II respectively. This was done by examining embryos for: 1) the number of times an embryo can show RC and at what stage of division; 2) the association with clinical factors such as age, type of ovarian stimulation regimen used (GnRH agonist or antagonist), insemination method, and quality of the original sperm sample in terms of origin, concentration and progressive motility; 3) the progress of embryo growth and grading on day 3; 4) the morphokinetics of embryo growth; and 5) the implantation rates of affected and unaffected embryos.

MATERIALS AND METHODS

This retrospective study was approved by the Research Ethics Committees at both the Joondalup Health Campus and Edith Cowan University, and informed consent was provided by each of the female patients and their partners. The study included 126 consecutive IVF/intracytoplasmic sperm injection (ICSI) treatment cycles at Fertility North from February to December 2013 undertaken by 117 women (aged 34.8 $\pm$ 4.5 years). A total of 789 embryos, including 353 originating from IVF insemination and 436 from ICSI, were cultured in the Embryoscope time-lapse system until 3 days after oocyte collection.

Controlled Ovarian Hyperstimulation and Gamete Preparation

Female patients underwent pituitary suppression by the administration of either the GnRH agonist Lucrin (Abbott Australasia; 10 IU for 1 week before menses then 6 IU to day of trigger) or Synarel (Pfizer Australia; 200 μg twice daily from 1 week before menses) or, starting on the 5th day of stimulation, the GnRH antagonist Orgalutran (Merck Sharp and Dohme [Australia]; 250 μg) or Cetrotide (Merck Serono [Australia]; 0.5 mg). This was followed by ovarian stimulation with recombinant FSH (Puregon, Schering-Plough; Gonal-f, Merck Serono). Follicles were aspirated and flushed with Flushing medium (Origio) with the use of ultrasound guidance 36 hours after the administration of hCG: 10,000 IU Pregnyl (Organon) or 500 IU Ovidrel (Merck Serono). Oocyte-cumulus complexes (OCC) collected were washed then cultured in Universal IVF (U-IVF) medium (Origio) at 6% CO_2 in air at 37°C. After the completion of oocyte collection, OCCs were transferred to U-IVF medium overlaid with paraffin oil (Origio) at 6% CO_2, 5% O_2, and 89% N_2 at 37°C for culture until either conventional IVF insemination 4–6 hours later or denudation in ICSI cases. Denudation was performed 2–4 hours after oocyte collection. OCCs were briefly exposed in Synvitro Hylase (Origio) for a maximum of 10 seconds, followed by mechanical removal of cumulus cells surrounding the oocytes. Denuded oocytes were then cultured in U-IVF medium covered by paraffin oil at 6% CO_2, 5% O_2 and 89% N_2, at 37°C for a minimum of 1 hour before ICSI insemination.

Sperm samples were collected with the use of surgical procedures or masturbation by the male partner within 2 hours of oocyte collection, after 2–4 days' sexual abstinence. Sperm were prepared by centrifuging samples through 95%:50% density gradients (Puresperm; Nidacon) and/or washing in U-IVF medium. Prepared sperm were cultured in U-IVF medium at 6% CO_2 in air at 37°C until use.

Insemination and Fertilization Check

In the IVF cases, prepared sperm were mixed with OCCs 4–6 hours after oocyte collection at a ratio of 50,000 sperm per oocyte, with up to five OCCs per dish. Mixed gametes were incubated at 6% CO_2, 5% O_2, and 89% N_2 at 37°C in a Minc incubator (Cook) overnight. Fertilization was confirmed by visualization of two pronuclei (2PN) 16–18 hours after insemination following removal of cumulus cells. Fertilized oocytes were then moved to wells containing 25 μL ISM1 medium (Origio) overlaid with 1.2 mL paraffin oil in Embryoslides (Unisense; Fertilitech), which had been preequilibrated for $\geq$16 hours. Oocytes were loaded individually into the microwells of the Embryoslides and then inserted into the Embryoscope (Unisense; Fertilitech) for culture and imaging.

In the ICSI cases, oocytes were transferred to the Embryoslides as described above immediately after the completion of sperm injection, then inserted into the Embryoscope for culture and imaging. Fertilization check on ICSI oocytes was performed with the use of Embryoviewer (Unisense; Fertilitech), the image analyzing software of the Embryoscope system, without having to remove them from culture.

Time-lapse Monitoring and Conventional Grading of Embryos

A total of 789 fertilized oocytes were cultured in the ISM1 medium in the Embryoscope imaging system at 6% CO_2, 5% O_2, and 89% N_2 at 37°C until day 3, with images taken every 10 minutes of each embryo at seven focal planes. Conventional morphology-based scoring of embryos was performed as described previously (1), with the use of images captured by the Embryoscope at 68 hours after insemination without considering time-lapse information. Embryos were scored from 1 to

4, with 1 being the best, based on cell count, symmetry, and degree of fragmentation. Morphokinetic analysis of embryos cultured in the Embryoscope imaging system was performed with the use of the Embryoviewer software. Each cleavage event of embryos was timed as the interval between insemination and the first observed moment of completion of corresponding event, e.g., t2 equals time taken from insemination to the complete separation of two daughter blastomeres. Cleavage timings were also analyzed relative to pronuclear fading (PNF) instead of insemination as a reference starting point. Morphokinetic parameters analyzed in the present study include t5/t5_pnf (time from insemination/PNF to 5 cells), t8/t8_pnf (time from insemination/PNF to 8 cells), s2 (time from 3 cells to 4 cells), s3 (time from 5 cells to 8 cells), and cc2 (time from 2 cells to 3 cells). Embryos with cc2 <5 hours were categorized as direct cleavage 2–3 cell and excluded from the morphokinetic comparisons (22). The presence of multinucleation in at least one blastomere was assessed at the 2- and 4- cell stages of each embryo. RC was defined as either: 1) two daughter blastomeres recombining after complete separation following cleavage division (type I; Supplemental Video 1; Supplemental Videos 1 and 2 are available online at www.fertstert.org); or 2) failed cytokinesis, the incomplete separation of blastomeres that then recombine fully (type II; Supplemental Video 2). Full annotation of a cohort of 8–10 day 3 embryos took <30 minutes to finish (including cleavage timings, multinucleation, embryo grading, and RC), and the time spent identifying RC on each embryo was <1 minute.

Embryo Transfer and Implantation Outcome Analysis

One or two embryos were selected for transfer at the end of culture, depending on the patient's age and request. A total of 121 treatment cycles resulted in embryo transfers, with the remaining five cycles having all embryos frozen to avoid ovarian hyperstimulation syndrome (OHSS). Viable implantation of embryos was confirmed by detection of a fetal heart beat on ultrasound at 7 weeks of pregnancy. The known implantation data (KID) (22) were used to calculate the proportion of transferred embryos that implanted and gave rise to a fetal heart beat for embryos showing RC or not.

Statistical Analysis

Proportion parameters were analyzed with the use of Fisher exact test. The predictive value of parameters on the occurrence of RC was analyzed via logistic regression, receiver operating characteristics (ROC) and area under the ROC curve (AUC). Continuous data (female age, t5, t8, s2, s3, cc2, and embryo score) were compared with the use of one-way analysis of variance and least significant difference post hoc tests. All statistical analysis was performed with the use of the Statistic Package for the Social Sciences 20.0 (SPSS). $P<.05$ was considered to be statistically significant.

RESULTS

Frequency and Pattern of RC

A total of 216/789 (27.4%) embryos showed evidence of RC at least once during the 3 days of culture, with 57 (26.4%) of these 216 embryos undergoing RC 2 or 3 times (Supplemental Fig. 1; Supplemental Figs. 1 and 2 are available online at www.fertstert.org). Of the 283 occasions of RC shown by these 216 embryos, Table 1 shows that the majority (232; 82.0%) were type II, namely, incomplete, with the complete form being in the minority (51; 18.0%). In addition, RC can occur in any of the three rounds of embryo cleavage, with 50/283 (17.7%) from 1 to 2 cells, 118/283 (41.7%) in 3- and 4-cell embryos, and 115/283 (40.6%) in embryos of 5 to 8 cells.

Factors Associated with RC

Logistic regression analysis showed that two main factors were significantly associated with increased occurrence of RC, namely ovarian stimulation with GnRH antagonists compared with agonists (odds ratio [OR] 1.683; $P=.005$) and ICSI compared with IVF (OR 1.600; $P=.004$), whereas female age was not a factor (OR 0.994; $P>.05$). Because ICSI is associated with sperm quality, the influence of sperm concentration and progressive sperm motility in embryos originating from ICSI were further examined with the use of ROC, demonstrating a negative predictive value for the occurrence of RC by the sperm progressive motility (AUC 0.573; $P=.021$), and this predictive value became stronger (AUC 0.644; $P=.005$) when considering only embryos originating from poor-motility sperm (<50%). The cutoff value for sperm progressive motility appeared to be optimal at 21%, although this value would still result in the detection of some false-positive results (specificity 0.593) and the missing of some true-positive results (sensitivity 0.683). However, there was no significant association between sperm concentration and RC in ICSI embryos (AUC 0.516; $P=.608$).

Table 2 presents ICSI embryos originating from men with surgically collected sperm or with different progressive sperm motility in the original semen sample (<21% as determined above, 21%–49%, and ≥50%). Embryos originating from poor-motility sperm (<21%) were associated with a significantly higher incidence of RC compared with those resulting from surgically collected sperm or suboptimal (21%–49%) or normal (≥50%) motility sperm (55.2% vs. 21.1%, 55.2% vs. 27.9%, and 55.2% vs. 29.5%, respectively; $P<.01$).

TABLE 1

The prevalence of the two types of reverse cleavage according to the cell cycle, being after the first mitotic division (2-cell embryo), the second round of mitotic divisions (3- and 4-cell embryos), or the third round of divisions (5- to 8-cell embryos).

	Reverse cleavage		
Round of mitotic division	Type I (complete)	Type II (incomplete)	Total
First	5	45	50 (17.7)
Second	24	94	118 (41.7)
Third	22	93	115 (40.6)
Total	51 (18.0)	232 (82.0)	283 (100)

Note: Data presented as n or n (%). A total of 216 embryos showed RC on 283 occasions, either type I (complete) or type II (incomplete).

Liu. Reverse cleavage of human embryos. Fertil Steril 2014.

TABLE 2

Incidence of reverse cleavage according to sperm parameters in 436 embryos generated after intracytoplasmic sperm injection.

		Sperm progressive motility in semen sample		
Stimulating protocol	**Surgically collected sperm**	**<21%**	**21%–49%**	**≥50%**
Agonist cycles	3/16 (18.8)[a]	5/5 (100)[a,b,c]	17/67 (25.4)[b]	13/58 (22.4)[c]
Antagonist cycles	9/41 (22.0)[a]	27/53 (50.9)[a,d]	31/105 (29.5)[d]	31/91 (34.1)
Total	12/57 (21.1)[e]	32/58 (52.2)[a,e,f]	48/172 (27.9)[f]	44/149 (29.5)[a]

Note: Data presented as n/n (%). Results are expressed according to sperm origin and the motility of the sperm in the original semen sample and the kind of GnRH analogue used in the ovarian stimulation protocol.
[a,b,c] Same superscript in the same row indicates statistically significant difference ($P<.01$).
[d] Same superscript in the same row indicates statistically significant difference ($P<.05$).
[e,f] Same superscript in the same row indicates statistically significant difference ($P<.001$).

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Analysis of the IVF cycles where all sperm parameters were within normal range (concentration ≥20 million/mL and progressive motility ≥50%) showed that RC was more prevalent among embryos derived from oocytes collected in stimulation cycles with a GnRH antagonist (67/257, 26.1%) than from cycles with a GnRH agonist (13/96, 13.5%; $P<.05$).

Progress and Fate of Embryos

Table 3 summarizes the grading and assessment of embryos. Embryos showing RC grew slower, as judged by the number with ≥6 cells by 68 hours after insemination, with only 103/216 (47.7%) embryos having ≥6 cells compared with 411/573 (71.7%) of embryos without RC ($P<.001$). The proportion of good-quality embryos on day 3 was significantly lower in those embryos showing RC (76/216, 32.5%) than in those not (337/573, 58.8%; $P<.05$). Morphology-based embryo score on day 3 was significantly worse in embryos with RC than those without (2.4 vs. 3.0; $P<.001$). Also, multinucleation at the 4-cell stage was more prevalent in RC embryos (16/158, 10.1%) than those without evidence of RC (25/501, 5.0%; $P<.05$). However, there was no difference in the occurrence of direct cleavage between the two groups (16.1% vs. 11.5%; $P>.05$).

Table 4 presents the woman's age and morphokinetic parameters (t5, t8, cc2, s2, and s3) for RC− and RC+ embryos that had been transferred. Embryos showing RC took longer to divide from the 3-cell to 4-cell and from 5-cell to 8-cell stages, having a significantly prolonged s2 and s3 compared with their RC− counterparts (both $P<.01$). Interestingly, the time taken to reach the 5-cell stage from insemination was not different between RC− embryos that implanted and those that did not. However, this did become clearer and significant when the time was expressed relative to PNF, which is a biologic reference point rather than a procedural one.

A total of 153 embryos with known implantation results from 121 embryo transfer cycles were included for analysis. RC− embryos implanted and progressed to a fetal heart beat significantly more frequently (29/131, 22.1%) than RC+ embryos (0/22; $P=.014$) (Supplemental Fig. 2). Although no fetal heart beats were detected after the transfer of RC embryos, one did result in biochemical pregnancy.

DISCUSSION

This study classified all RC-affected embryos into two types based on whether the initial cleavage attempt achieved complete cell separation (type I) or progressed only to a "figure of 8" shape (type II) before rejoining, examples of which can be seen online (Supplemental Videos 1 and 2). Type II was seen far more frequently than type I (Table 1). Earlier descriptions of RC described only the complete form (24) and this partly explains the higher incidence of RC described in the present study. The failure of any RC embryos to implant and progress to a clinical pregnancy in this study supports the notion that both forms of RC have a significant impact on embryo implantation potential, and the possibility of type II embryo blastomeres separating during the 10 minutes between video

TABLE 3

Comparisons of embryo development, conventional grading, and multinucleation status between embryos with (RC+) and without (RC−) reverse cleavage.

		Embryos with and without reverse cleavage	
Embryo feature	**Parameter**	**RC+ (n = 216)**	**RC− (n = 573)**
Rate of development	≥6-cell on day 3	103 (47.7)[a]	411 (71.7)[a]
Quality	Good	76 (35.2)[b]	337 (58.8)[b]
Grading	Embryo score (1 [best] to 4 [worst])	3.0 ± 1.1[a]	2.4 ± 1.3[a]
Multinucleation	At 2-cell	45/199 (22.6)	102/557 (18.3)
	At 4-cell	16/158 (10.1)[b]	25/501(5.0)[b]

Note: Data presented as n (%), mean ± SD, or n/n (%).
[a] Same superscript in same row indicates statistically significant difference ($P<.001$).
[b] Same superscript in same row indicates statistically significant difference ($P<.05$).

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TABLE 4

Woman's age and time (h) of various morphokinetic parameters in transferred embryos with reverse cleavage (RC+) or not (RC−), and of known implantation history resulting in a fetal heart beat or not.

	Not implanted		Implanted
	RC+	RC−	RC−
Age (y)	35.0 ± 3.6 (21)	35.7 ± 4.3 (96)[a]	32.8 ± 5.2 (29)[a]
t5	49.8 ± 6.3 (21)	50.6 ± 4.8 (91)	48.7 ± 3.8 (29)
t5_pnf	26.4 ± 6.3 (21)	27.3 ± 2.9 (91)[a]	25.2 ± 2.4 (29)[a]
cc2	11.1 ± 1.3 (21)	11.5 ± 2.3 (96)	10.9 ± 0.9 (29)
s2	4.1 ± 5.8 (21)[b,c]	1.1 ± 2.3 (95)[b]	0.3 ± 0.4 (28)[c]
t8	55.0 ± 6.0 (9)	53.3 ± 4.6 (65)	52.3 ± 3.5 (25)
t8_pnf	31.5 ± 6.1 (9)	30.4 ± 4.1 (65)	28.7 ± 2.1 (25)
s3	9.5 ± 7.4 (9)[a,b]	3.8 ± 3.8 (65)[b]	3.8 ± 3.3 (25)[a]

Note: Embryos with direct cleavage (cc <5 h) were excluded from comparisons. Values are expressed as mean ± SD (n).
[a] Same superscript in same row indicates statistically significant difference ($P< .01$).
[b,c] Same superscript in same row indicates statistically significant difference ($P< .001$).

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frames can not be excluded. The occurrence of repeated RC in over one-fourth of embryos showing the phenomenon was not anticipated based on the one meeting abstract in the literature (24), and the present study demonstrates that RC can occur at any stage of cell division up to the 8-cell stage.

RC did not appear to be associated with the woman's age at the time of the egg collection, but with the regimen used for ovarian stimulation, with RC seen more frequently in cycles with the use of GnRH antagonists than those with GnRH agonists. Although no conclusive data are available from earlier studies comparing embryology and clinical results following cycles using these two ovarian-stimulating protocols (24, 26–30), the two regimens did not seem to be equivalent for different patient groups (31–34). The impact on the ovary and hence the oocyte is also implied indirectly with the follicular fluid concentrations of insulin-like growth factors and their binding proteins being different following the different protocols (35). Further larger-scale investigation considering detailed cycle characteristics, such as levels of associated hormones, would be desirable.

The occurrence of RC by embryos is not restricted to the female contribution, with reduced progressive sperm motility in the semen sample produced on the day of egg collection (which often results in the selection of IVF or ICSI) related to a higher incidence of RC (Table 2). Multinucleation was also seen in the present study to be more common by the 4-cell stage in RC+ embryos, and this has been reported previously to have an adverse impact on subsequent implantation (36, 37). One possible mechanism of multinucleation is karyokinesis in the absence of cytokinesis (38), which is also implicated in male-factor infertility because multinucleation is seen to be more prevalent when the man's semen has an increase in sperm DNA damage (39). An example of the formation of multinucleation following RC is shown in Supplemental Video 1. Interestingly, surgically collected sperm from men with azoospermia or severe oligozoospermia resulted in prevalence of RC similar to ejaculated sperm with good motility (Table 2). However, further investigation is required to determine if bypassing ejaculation and collection of sperm surgically from the male reproductive tract is helpful.

Embryos showing RC do seem to be compromised compared with their RC-unaffected counterparts. This is revealed in both the reduced proportion that reach ≥6 cells by day 3 (Table 3), as would be evident with the use of conventional embryo scoring, and the slower times taken to divide from 3-cell to 4-cell and from 5-cell to 8-cell stages, as presented in Table 4. Among timing parameters measured during the early cleavage stages, t5, cc2, and s2 are thought to be the most predictive and have been used to form a morphokinetic algorithm for improved embryo selection (20). The results of the present study suggest that any early cell-cleavage algorithm generated with time-lapse videography should include RC. The use of time-lapse videography reveals abnormalities of embryo division associated with reduced implantation potential, such as RC and direct cleavage (22), and these phenomena are not able to be seen with the use of conventional static observations.

The present study is limited to the use of one particular type of time-lapse system, the Embryoscope. Although other time-lapse systems would presumably be able to detect these abnormalities, this requires confirmation. The compromised growth and development of embryos with RC in the present study is further supported by the known implantation data (Supplemental Fig. 2). Although larger studies are required to confirm this, it is likely that these embryos should be given lower priority at selection for transfer. The culture of embryos did not go beyond day 3, because the clinic operates an early-cleavage transfer program, so the ability of embryos showing RC to form blastocysts could not be determined.

In summary, the present study showed significantly compromised growth and development of embryos with RC, culminating in poor implantation potential. The phenomenon can occur at any stage during the first 3 days of embryo culture, and embryos can show it on more than one occasion. Related factors are not restricted to those affecting the oocyte, but also include sperm factors reflected by poor progressive sperm motility.

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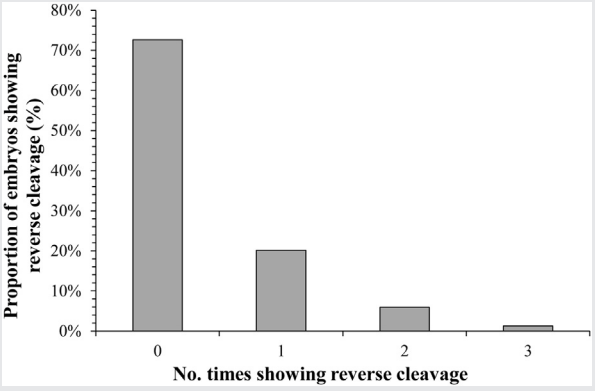
REFERENCES

1. Alpha Scientists in Reproductive Medicine, ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Hum Reprod 2011;26:1270–83.
2. Baczkowski T, Kurzawa R, Glabowski W. Methods of embryo scoring in in vitro fertilization. Reprod Biol 2004;4:5–22.
3. Scott L, Finn A, O'Leary T, McLellan S, Hill J. Morphologic parameters of early cleavage-stage embryos that correlate with fetal development and delivery: prospective and applied data for increased pregnancy rates. Hum Reprod 2007;22:230–40.
4. Machtinger R, Racowsky C. Morphological systems of human embryo assessment and clinical evidence. Reprod Biomed Online 2013;26:210–21.
5. Siristatidis C, Hamilton M. Single embryo transfer. Obstet Gynaecol Reprod Med 2007;17:192–4.
6. Montag M, Toth B, Strowitzki T. New approaches to embryo selection. Reprod Biomed Online 2013;27:539–46.
7. Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J, Hesla J. A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. Hum Reprod 1998;13:3434–40.

8. Zander-Fox D, Tremellen K, Lane M. Single blastocyst embryo transfer maintains comparable pregnancy rates to double cleavage-stage embryo transfer but results in healthier pregnancy outcomes. Aust N Z J Obstet Gynaecol 2011;51:406–10.
9. Fernando D, Halliday JL, Breheny S, Healy DL. Outcomes of singleton births after blastocyst versus nonblastocyst transfer in assisted reproductive technology. Fertil Steril 2012;97:579–84.
10. Kallen B, Finnstrom O, Lindam A, Nilsson E, Nygren KG, Olausson PO. Blastocyst versus cleavage stage transfer in in vitro fertilization: differences in neonatal outcome? Fertil Steril 2010;94:1680–3.
11. Kalra SK, Ratcliffe SJ, Barnhart KT, Coutifaris C. Extended embryo culture and an increased risk of preterm delivery. Obstet Gynecol 2012;120:69–75.
12. Diamond MP, Willman S, Chenette P, Cedars MI. The clinical need for a method of identification of embryos destined to become a blastocyst in assisted reproductive technology cycles. J Assist Reprod Genet 2012;29:391–6.
13. Dar S, Lazer T, Shah PS, Librach CL. Neonatal outcomes among singleton births after blastocyst versus cleavage stage embryo transfer: a systematic review and meta-analysis. Hum Reprod Update 2014;20:439–48.
14. Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, et al. Noninvasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. Nat Biotechnol 2010;28:1115–21.
15. Herrero J, Tejera A, Albert C, Vidal C, de los Santos MJ, Meseguer M. A time to look back: analysis of morphokinetic characteristics of human embryo development. Fertil Steril 2013;100:1602–9.e1–4.
16. Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boostanfar R, et al. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: results from a prospective multicenter trial. Fertil Steril 2013;100:412–9.e5.
17. Chen AA, Tan L, Suraj V, Reijo Pera R, Shen S. Biomarkers identified with time-lapse imaging: discovery, validation, and practical application. Fertil Steril 2013;99:1035–43.
18. Kirkegaard K, Agerholm IE, Ingerslev HJ. Time-lapse monitoring as a tool for clinical embryo assessment. Hum Reprod 2012;27:1277–85.
19. Dal Canto M, Coticchio G, Mignini Renzini M, De Ponti E, Novara PV, Brambillasca F, et al. Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation. Reprod Biomed Online 2012;25:474–80.
20. Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB, Remohi J. The use of morphokinetics as a predictor of embryo implantation. Hum Reprod 2011;26:2658–71.
21. Hlinka D, Kalatova B, Uhrinova I, Dolinska S, Rutarova J, Rezacova J, et al. Time-lapse cleavage rating predicts human embryo viability. Physiol Res 2012;61:513–25.
22. Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escriba MJ, et al. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. Fertil Steril 2012;98:1458–63.
23. Athayde Wirka K, Chen AA, Conaghan J, Ivani K, Gvakharia M, Behr B, et al. Atypical embryo phenotypes identified by time-lapse microscopy: high prevalence and association with embryo development. Fertil Steril 2014;101:1637–48.e1–5.
24. Hickman CF, Campbell A, Duffy S, Fishel S. Reverse cleavage: its significance with regards to human embryo morhpokinetics, ploidy and stimulation protocol. Hum Reprod 2012;27(Suppl 2):ii103–5.
25. Montag M, Pedersen KS, Ramsing N. Time-lapse imaging of embryo development: using morphokinetic analysis to select viable embryos. In: Quinn P, editor. Culture media, solutions, and systems in human ART. Cambridge: Cambridge University Press; 2014:222–3.
26. Maldonado LG, Franco JG Jr, Setti AS, Iaconelli A Jr, Borges E Jr. Cost-effectiveness comparison between pituitary down-regulation with a gonadotropin-releasing hormone agonist short regimen on alternate days and an antagonist protocol for assisted fertilization treatments. Fertil Steril 2013;99:1615–22.
27. Luna M, Vela G, McDonald CA, Copperman AB. Results with GnRH antagonist protocols are equivalent to GnRH agonist protocols in comparable patient populations. J Reprod Med 2012;57:123–8.
28. Orvieto R, Meltzer S, Rabinson J, Zohav E, Anteby EY, Nahum R. GnRH agonist versus GnRH antagonist in ovarian stimulation: the role of endometrial receptivity. Fertil Steril 2008;90:1294–6.
29. Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. J Clin Endocrinol Metab 2003;88:166–73.
30. Bahceci M, Ulug U, Sismanoglu A, Tosun S, Cengiz B. Early pregnancy loss rates were different among singleton gestations conceived by ICSI using GnRH agonist and antagonist. J Assist Reprod Genet 2009;26:227–9.
31. Check JH, Choe JK, Cohen R, Wilson C. A study to determine the efficacy of controlled ovarian hyperstimulation regimen using a gonadotropin releasing hormone agonist versus antagonist in women of advanced reproductive age with varying degrees of oocyte reserve on outcome following in vitro fertilization–embryo transfer. Clin Exp Obstet Gynecol 2013;40:191–2.
32. Kumbak B, Akbas H, Sahin L, Karlikaya G, Karagozoglu H, Kahraman S. Ovarian stimulation in women with high and low body mass index: GnRH agonist versus GnRH antagonist. Reprod Biomed Online 2010;20:314–9.
33. Lin H, Li Y, Li L, Wang W, Yang D, Zhang Q. Is a GnRH antagonist protocol better in PCOS patients? A meta-analysis of RCTs. PLoS One 2014;9:e91796.
34. Kurzawa R, Ciepiela P, Baczkowski T, Safranow K, Brelik P. Comparison of embryological and clinical outcome in GnRH antagonist vs. GnRH agonist protocols for in vitro fertilization in PCOS nonobese patients. A prospective randomized study. J Assist Reprod Genet 2008;25:365–74.
35. Choi YS, Ku SY, Jee BC, Suh CS, Choi YM, Kim JG, et al. Comparison of follicular fluid IGF-I, IGF-II, IGFBP-3, IGFBP-4 and PAPP-A concentrations and their ratios between GnRH agonist and GnRH antagonist protocols for controlled ovarian stimulation in IVF–embryo transfer patients. Hum Reprod 2006;21:2015–21.
36. Van Royen E, Mangelschots K, Vercruyssen M, De Neubourg D, Valkenburg M, Ryckaert G, et al. Multinucleation in cleavage stage embryos. Hum Reprod 2003;18:1062–9.
37. Pelinck MJ, De Vos M, Dekens M, Van der Elst J, De Sutter P, Dhont M. Embryos cultured in vitro with multinucleated blastomeres have poor implantation potential in human in-vitro fertilization and intracytoplasmic sperm injection. Hum Reprod 1998;13:960–3.
38. Pickering SJ, Taylor A, Johnson MH, Braude PR. An analysis of multinucleated blastomere formation in human embryos. Hum Reprod 1995;10:1912–22.
39. Zini A, Meriano J, Kader K, Jarvi K, Laskin CA, Cadesky K. Potential adverse effect of sperm DNA damage on embryo quality after ICSI. Hum Reprod 2005;20:3476–80.

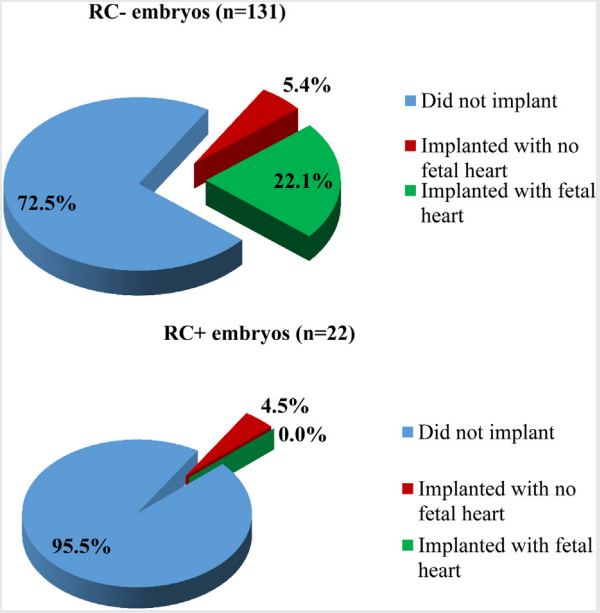
SUPPLEMENTAL FIGURE 1



The proportion of all 789 embryos that showed reverse cleavage never, once, twice, or three times.

Liu. Reverse cleavage of human embryos. Fertil Steril 2014.

SUPPLEMENTAL FIGURE 2



Known implantation results following the transfers of embryos with (RC+) or without (RC−) reverse cleavage.

Liu. Reverse cleavage of human embryos. Fertil Steril 2014.