A multicenter prospective study to assess the effect of early cleavage on embryo quality, implantation, and live-birth rate

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Objective: To investigate the impact of early cleavage (EC) on embryo quality, implantation, and live-birth rates.
Design: Prospective cross-sectional study.
Setting: Multicenter study.
Patient(s): Seven hundred embryo transfers and 1,028 early-stage human embryos.
Intervention(s): None.
Main Outcome Measure(s): Implantation according to the presence of EC and embryo quality.
Result(s): The presence of EC is associated with embryo quality, especially in cycles with autologous oocytes. However, the use of EC as an additional criterion for selecting an embryo for transfer does not appear to significantly improve likelihood of implantation. Furthermore, embryos that presented EC had live-birth rates per implanted embryo similar to those that did not show any sign of cleavage.
Conclusion(s): At least for conventional embryo culture and morphologic evaluations, the additional evaluation of EC in embryos may not be valuable to improve embryo implantation. (Fertil Steril® 2014;101:981–7. ©2014 by American Society for Reproductive Medicine.)
Key Words: Early cleavage, embryo quality, implantation, live-birth rates

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Early cleavage, understood as the first embryo mitosis at 25–27 hours after insemination, has been considered to be an embryo quality parameter (1–9). Over the past decade, numerous studies have associated its presence with embryonic morphology on days 2 and 3 (1–4), development until the blastocyst stage (5), chromosome anomalies (6), embryo viability (7–9), implantation rate (2, 10), and abortion rate (11). However, the conclusions drawn are too contradictory to establish their use. Despite that, many publications advise using early cleavage (EC) as a "secondary parameter" to decide between embryos of similar quality.

More recently, however, time-lapse studies demonstrate that the EC time variable does not have sufficient predictive value to help embryo selection (12). Therefore, other more novel variables, such as first cytokinesis duration (13), the time when the embryo has five cells, or the synchrony between the second and third mitotic embryo cleavage, seem to be more important when predicting evolution for the blastocyst stage. Strangely enough, they are unable to forecast blastocyst morphology.

The Istanbul consensus group leaves to the laboratory the decision of whether or no to include the EC

variable in embryo selection (14). In this context, the Spanish Association of Reproduction Biology Studies (ASEBIR) considered conducting a multicenter study with several Spanish centers to evaluate the effect of this variable on embryo quality and implantation capacity to add, or not, the use of this variable to our recommendations for embryo selection.

MATERIALS AND METHODS
Study Patients

A multicenter prospective study, promoted by ASEBIR, was carried out from January to June 2011. Twenty centers initially participated in this study, which included 780 embryo transfers and 2,076 embryos. The participation of all interested centers was anonymously requested through the ASEBIR website and e-mail address. Institutional Review Board approval was obtained. The inclusion criteria were first or second in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles with autologous or donor oocytes. Implantation rates were calculated from those embryos originating from cycles with a 100% or a 0% implantation rate, or from homogenous embryo transfers for EC, that is to say, those embryos which, despite presenting a different evolution on later embryo development days, were similar in morphology terms when considering the EC parameter. Embryo transfers were done on both day 2 and day 3. After eliminating any incorrectly enterered implantation data, the sample size was 700 transfers and 1,028 embryos with identified implantation.

Evaluating Early Cleavage

The EC parameter was established at 25–27 hours after insemination by determining the following stages: visible pronuclei, syngamy, or 2- or 3-cell cleavage.

In this interval, the embryos with two cells were classified as EC embryos, and could present two cells or more (2C, >2C). Those that had not divided were classified as non–early-cleavage embryos (Non-EC).

Day 2 and Day 3 Embryo Morphology

On days 2 and 3, embryos were evaluated at 43–45 hours and 63–65 hours after IVF or ICSI, respectively. Embryo quality was determined based on the number of blastomeres (0, 1, 2, 3, 4, 5, 6, >6), the percentage of fragmentation (<10%, 11%–25%, 26%–35%, >35%), blastomere symmetry (equal, similar, different), vacuoles (absent, scarce and/or diameter <5 μm, abundant), the zona pellucida (normal;-abnormal), and the presence of multinucleated cells. The day 2 embryos were classified into four categories (A, B, C, D), where category A gave the best and category D the worst prognosis for a combination of the various aforementioned morphologic parameters. To classify the day 3 embryos, all four categories were assigned according to the evolution of the embryos from day 2 to day 3 (14).

Culture Conditions System

Embryo culture was performed under CO_2 concentration ranging from 5% to 6% CO_2 in air. Three different types of culture media were also used: Global, Sage, and Vitrolife.

Statistical Analysis

In order to determine variability among the participating centers, the groups participating in the multicenter study first did an external consistency test to evaluate the homogeneity among groups regarding fertilization, EC, and the ASEBIR morphologic classification.

All of the centers assessed a video containing 25 films on the embryonic development of 25 embryos from ICSI to 65 hours after ICSI. This video stated the time since insemination so that the participating users could analyze the images within the requested time ranges; based on this, fertilization was evaluated, as were the embryonic evolution parameters (i.e., EC) and the remaining embryo morphological parameters on days 2 and 3 (number of cells, fragmentation, symmetry, vacuoles, zona pellucida, and multinucleation). This video came with a data collection document in which the embryonic evaluation data were stored. The ranges set to observe different events were 17–19 hours for fertilization, 25–27 hours for EC, 43–45 hours for day 2, and 63–65 hours for day 3. The consistency index among the participating centers for all of the evaluations was measured by kappa statistics. Values of ≥0.6 were considered to be good.

To make a comparison between the groups of dichotomous variables, a χ^2 test was used. Implantation rates were expressed as percentage probabilities with 95% confidence interval (CI). The effect of other covariates (i.e., the ASEBIR embryo scoring system, day of embryo transfer, age range, and oocyte insemination type) on implantation was assessed by a forward logistical regression analysis. A power analysis calculation for the raw EC data was also performed by means of the Statistical Power Calculator Tool Kit on the DSS Research web page (www.dssresearch.com/KnowledgeCenter/toolkitcalculators/statisticalpowercalculators.aspx.)

RESULTS

The etiologies of the studied cycles were distributed as follows: age in 27.2%, endometriosis in 7.1%, infertility of unknown origin in 23.7%, male factor in 28.6%, tubal factor in 4.2%, ovarian failure in 1.9%, and polycystic ovary in 5.3%. The insemination techniques used were IVF in 7.2%, ICSI in 77.8%, and mixed IVF/ICSI in 14.3%.

Of all the transfers, 35.9% (n = 251) corresponded to day 2 and 64.1% (n = 449) to day 3; 29.6% (n = 207) of the embryo transfers were done with one embryo, 63.7% (n = 446) with two embryos, and 6.7% (n = 47) with three. Of all 700 transfers, 443 were cycles with autologous oocytes and 257 with donor oocytes.

Measuring Consistency among Centers

After evaluating each analyzed parameter, a satisfactory consistency index for each consulted parameter was found among the groups: fertilization, EC, and ASEBIR embryo

score (Fig. 1). Only center 2 had no acceptable kappa value and was not considered for the implantation calculations.

Distribution of Embryos According to EC in Patients with Autologous Oocytes

Of the 1,679 embryos of the own oocyte cases studied, 22.0% showed signs of EC. Table 1 provides the ASEBIR category distribution with the day 2 embryos in the patients with autologous oocytes. Significantly more embryos in the best category (A) were found among the embryos showing signs of EC (51.1%) compared with the Non-EC ones (38.7%). For the day 3 embryos, differences in the distribution of the various embryo categories were observed. The percentage of optimum embryos when EC was observed was 38.8% compared with 34.0% in those cases preceded by Non-EC.

Distribution of Embryos According to EC in Patients with Donor Oocytes

The embryos from donated oocytes included a significantly higher proportion of embryos undergoing EC. Of the 379 embryos studied in the donor oocytes group, 32.2% showed signs of EC. Notwithstanding, no impact on embryo quality on day 2 or 3 was observed in the egg donation cycles. A similar proportion of category A embryos was detected among the embryos undergoing EC from both day 2 and day 3 embryos (66.9% vs. 69.4% and 64.8% vs. 57.4%, respectively).

Embryo Implantation with Autologous Oocytes

As Table 2 indicates, when analyzing the presence of EC as a single isolated parameter, the implantation rates in the autologous oocytes group were higher in the EC embryos: 31.2% versus 22.6% of the non-EC cases. The statistical power of the present study was 86.6%.

When stratifying for each ASEBIR category, we observed that the selection of optimum embryos for transfer eliminated the EC effect on implantation. Evaluation of EC seemed to be effective only for the embryos in the inferior morphologic category. For example, the embryos of categories B and C that underwent EC implanted in 26.2% and 25.7% of the cases, respectively, compared with 16.5% and 14.9%, respectively, when EC did not occur. These differences were statistically significant only for embryos classified as category C (Table 2).

When considering age alone, the evaluation of EC proved to be more useful for implantation rates in patients aged ≤35 years; (42.9%; 95% CI [30.8%–55.8%] in the EC group versus 28.4%; 95% CI [17.9%–38.9%] in the Non-EC group), whereas EC did not affect the implantation rates in women over the age of 35 years (17.6%; 95% CI [11.9%–29.9%] in the EC group versus 28.4%; 95% CI [17.9%–38.9%] in the Non-EC group). Nonetheless, when both the age range and the ASEBIR categories were added to the linear regression analysis model, age did not change the odds ratio (OR) of EC. The explanation for this is that the age range and the distribution of the ASEBIR embryo categories were similar in our sample population ($P = .119$).

Additionally, the type of inseminations was also evaluated; some authors have shown that early cleavage is an independent predictor of birth in ICSI, but not in IVF, patients (4).

As a matter of fact, the forward linear regression analysis showed that only embryo quality and no other variables included in the analysis was able to modify the OR of EC for implantation by more than 10%, which changed from OR 0.791 (95% CI 1.268–2.529) to OR 1.413 (95% CI 0.982–2.034) when the ASEBIR categories were introduced. Therefore, the possibility of selecting embryos by morphology diminished the prediction of EC for implantation. Actually, the Nagerlkerke-corrected R^2 value showed that the ASEBIR embryo score over EC was a better implantation model ($R^2 = 0.066$ vs. $R^2 = 0.134$).

Embryo Implantation in Cycles of Egg Donation

For the egg donation cycles (Table 2), EC alone was unable to predict implantation (42.5% vs. 36.6%, respectively). This lack of difference is probably due to the fact that most transferred embryos were actually top-quality ones. These results are in line with those presented in the cycles with autologous oocytes.

Presence of Multinucleation Signs in EC Embryos

We also studied multinucleation in all of the analyzed embryos. We found that this phenomenon appeared in 10.9% of embryos with two cells and in 7% of those with three cells. The overall implantation rate for this type of embryos was 24.2% compared with 36.5% overall implantation of non-multinucleated embryos with EC.

Live-Birth Rates According to EC

From the 303 implanted embryos, we were able to obtain information for a total of 301 embryos. Forty-six first-trimester abortions were observed and 211 live-births registered. Four twin pregnancies were reported: three in the Non-EC group and one in the EC group. The live-birth rates per implanted embryo according to EC stage was also calculated. Embryos that presented EC had similar live-birth rate per embryo as the ones that did not show any sign of cleavage (67.9% vs. 72.0%; Table 3).

DISCUSSION

To date, this is the first multicenter prospective study to evaluate EC as an indicator of embryo quality and implantation capacity in accordance with the quality of embryos on days 2 and 3 in own-oocyte and donor-oocyte patient groups. Given the multicenter nature of this study, special emphasis was placed on evaluating the homogeneity index among the different groups participating to control variability among centers. Kappa indices were calculated for fertilization, identification of EC, and embryo quality according to the ASEBIR score system.

After the analysis, all except one center fell within the permitted ranges, i.e., values ≥0.6, thus demonstrating the homogeneity and robustness of the study (15). Interestingly,

FIGURE 1
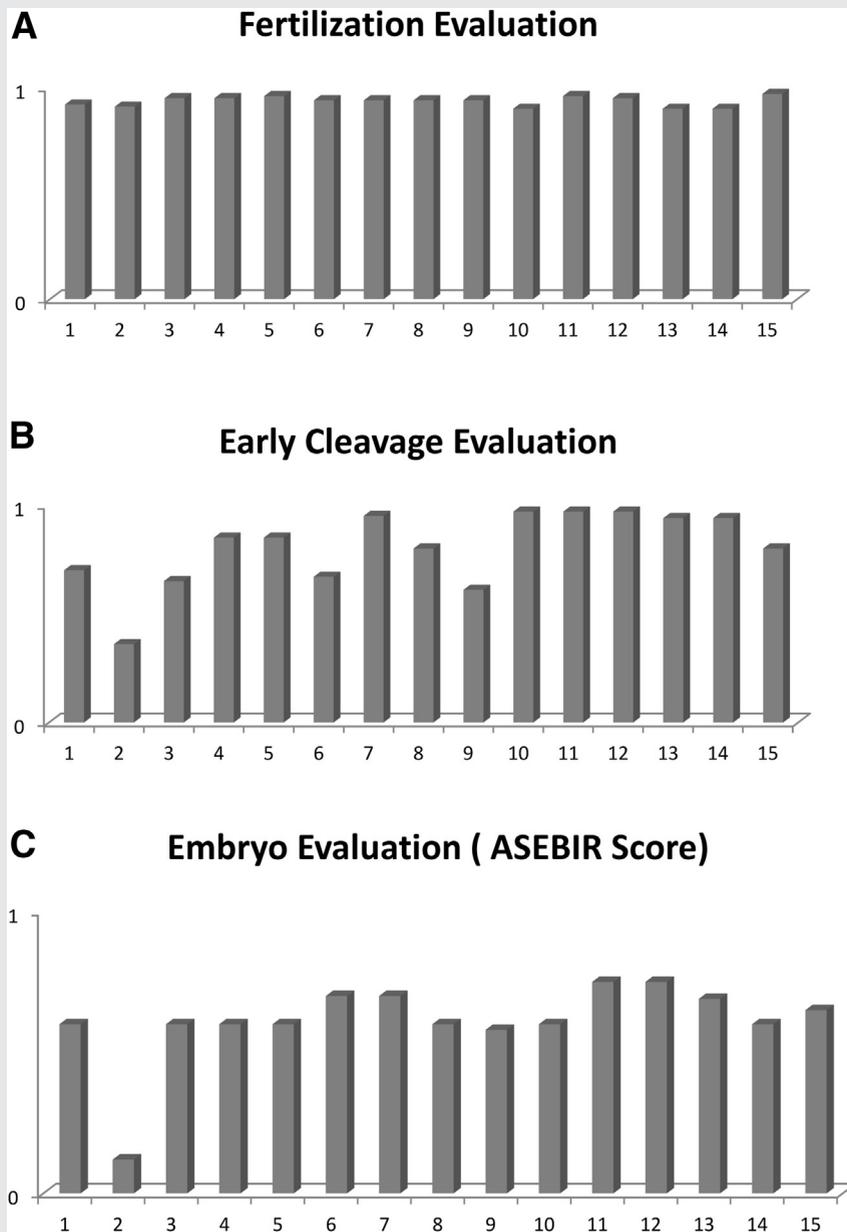
Kappa index parameters for (A) fertilization, (B) early cleavage, and (C) ASEBIR embryo score evaluation calculated for the centers participating in the study. The y axis shows the kappa index value for each center. Centers with values <0.6 were not considered for implantation calculations.

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EC and ASEBIR embryo score, compared with fertilization evaluation, obtained lower consistency values in all the centers. However, they fell above the 0.6 value cutoff. The center that did not reach the minimal EC kappa value also presented a very poor kappa value for the ASEBIR embryo score (Fig. 1C).

The time interval selected for the study was from 25 to 27 hours after insemination, this being the range most frequently used in the majority of studies (16, 17).

Many articles in the literature deal with the importance of taking EC into account to improve embryo selection before transfer and to help reduce multiple pregnancies. Neverthe-

less, there is some discrepancy as to the use of its evaluation. Some groups have observed an association with not only the quality of embryos on days 2 and 3, but also with rates of pregnancy and ongoing pregnancy when EC embryos were transferred (3, 4, 8, 9, 18, 19). For example, the percentage of optimum embryos in early stages went from 62.5% for EC embryos within 25–27 hours after insemination to 33.4% for non-EC embryos (4). Similarly, other groups have observed that the developmental capacity at more advanced stages also was associated with the presence or absence of EC. For example, 32.2% and 18.0% of the embryos undergoing EC, respectively, reached the expanding and hatching

TABLE 1

Distribution of embryo quality according to early cleavage (EC) and the origin of oocytes.

	Embryo quality			
	A	**B**	**C**	**D**
Autologous oocytes				
Day 2				
EC	252/493[a,b] (51.1%)	107/493 (21.7%)	94/493 (19.3%)	40/493 (8.1%)
Non-EC	211/544[a,b] (38.7%)	144/544 (26.4%)	136/544 (25.0%)	53/544 (9.7%)
Day 3				
EC	118/304[a] (38.8%)	72/304 (23.6%)	63/304 (20.7%)	51/304 (16.7%)
Non-EC	115/338[a] (34.0%)	68/338 (20.1%)	87/338 (25.7%)	68/338 (20.1%)
Egg donor oocytes				
Day 2				
EC	67/101[a] (66.9%)	20/101 (19.9%)	4/101 (3.9%)	10/101 (9.9%)
Non-EC	75/108[a] (69.4%)	14/108 (12.9%)	17/108 (15.7%)	2/108 (1.8%)
Day 3				
EC	61/94[a] (64.8%)	23/94 (24.4%)	5/94 (5.3%)	5/94 (5.3%)
Non-EC	54/94[a] (57.4%)	16/94 (17.0%)	18/94 (19.1%)	6/94 (6.3%)

Note: $P < .001$.
[a] Statistical difference in relation to the embryo categories in the same row.
[b] Statistical difference between EC and Non-EC groups.

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blastocyst stages compared with 16.6% and 7.1% of Non-EC embryos (18). This improvement for early-stage embryo quality and evolution capacity to reach the blastocyst stage has been repeated in most works, though a few studies have not found this relation (11, 20).

A forward logistic regression analysis was done and included the four categories (A, B, C, D) of the ASEBIR scoring system, as well as the day of embryo transfer, age range, and oocyte insemination type. Based on this analysis, the OR of EC was affected only by embryo quality. This means that neither the day of transfer nor the insemination type modifies the EC effect on implantation more than embryo quality at the time of the embryo transfer.

For this reason, the impact of EC on implantation capacity is unclear when stratifying groups according to the quality of transferred embryos. This is a very important aspect to consider, because if most of the embryos that undergo mitosis at 25–27 hours after insemination are actually embryos with a good morphology on day 2 or 3, it seems to be senseless to add another assessment to the embryo evaluation. If we contemplate not only the risk benefit of this strategy, but also the introduction of another evaluation on day 1, some disadvantages emerge; incubators would be increasingly opened,

which implies repercussions on changes in temperature and CO_2 concentration; and it would imply heavier loads for laboratories as more time would be spent on the new evaluation. These drawbacks are overcome by implementing certain available laboratory image analysis techniques, which are increasingly used in laboratories. Unfortunately, they continue to be expensive and not all laboratories can afford them.

This coincides with the results presented by Giorgetti et al. in their prospective study using 193 single-embryo transfer (SET) cycles. Those authors concluded that when a transfer is performed with good-quality embryos, EC offers no benefit (17). That agrees with the results of another prospective study with 196 cycles, young women (<36 years) and SET, that reported a 27.6% delivery rate for the group of embryos in which an EC evaluation was done. This result was similar to the 24.5% rate obtained when EC was not evaluated (21). Similarly, EC did not affect the implantation rates obtained in our study when variables such as embryo quality or age come into play. In our study, this situation occurred for both autologous and donor oocytes.

Nowadays, access to the vast quantity of data that image analysis studies generate can provide new data on the

TABLE 2

Implantation rates (% [95% confidence interval]) according to embryo quality, state of early cleavage (EC), and origin of oocytes.

ASEBIR category	Autologous oocytes		P value	Donor oocytes		P value
	EC	Non- EC		EC	Non- EC	
A	70/182 (38.5% [50.7–26.3])	54/136 (39.7% [52.1–27.3])	ns	37/83 (44.6% [57.8–31.4])	22/63 (34.9% [46.6–22.3])	ns
B	27/103 (26.2% [36.3–16.1])	15/91 (16.5% [24.5–8.5])	ns	8/17 (47.1% [60.9–33.3])	13/23 (56.5% [71.6–41.5])	ns
C	18/70 (25.7% [35.7–15.7])	17/114 (14.9% [22.5–7.3])	<.05	3/8 (37.5% [50.2–24.8])	5/18 (27.8% [38.4–17.2])	ns
D	4/23 (17.4% [25.7–9.1])	9/80 (11.3% [17.9–4.7])	ns	0/5 (0% [–])	1/6 (16.7% [25.3–8.1])	ns
Total	119/382 (31.2% [42.2–20.2])	95/421 (22.6% [31.9–13.3])	<.05	48/113 (42.5% [55.3–29.7])	41/112 (36.6% [48.5–24.7])	ns

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TABLE 3

Abortion and live-birth rates (% [95% CI]) per implanted embryo according to cleavage stage of the embryos at 25–27 hours after insemination.

Cleavage stage	1st-trimester abortion rate/embryo	Live-birth rate/embryo
EC	23/176 (13.1% [8.1%–18.1%])	121/176 (68.7% [61.8%–75.6%])
Non-EC	23/125 (18.4% [15.4%–30.0%])	90/125 (72.0% [64.1%–79.8%])

Note: No significant differences were observed between groups. CI = confidence interval; EC = early cleavage.

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relation between EC and embryo quality, and its relation with implantation. In fact, more than EC, other variables, such as direct 3-cell cleavage, have a determinant value to predict chromosome anomalies (6) and minimum implantation rates (22).

Morphokinetics studies with the use of time-lapse technology have revealed that for the embryos reached on day 2 with four or more cells, the pronuclei disappear early and that EC advances (23). This relation seems to be mitigated in more advanced stages of embryo development when other variables, such as embryo transcription activation, might affect development (13, 24, 25). Indeed, more recent morphokinetic analyses with the use of time-lapse technology in ovum-donation cycles show that although the first embryo mitotic division occurs significantly earlier in those embryos that develop to the blastocyst stage (26.8 ± 0.2) than in those that do not (27.9 ± 0.5), this variable is not capable of distinguishing their quality (26). In the present study, EC does not appear to be a determinant factor in embryo selection for improving either implantation rates or live-birth rates.

In the study by Wong et al. (13) with 100 thawed embryos, they did not expect the EC variable to be calculated, given the nature of the embryos under study, which were frozen zygotes donated for research purposes. Therefore it was not feasible to do division calculations in relation to insemination time. In that study, other variables, such as duration of first cytokinesis (0–33 min), the time between the first and second divisions (7.8–14.3 h), and synchrony in the 4-cell stage (0–5.8 h), showed high sensitivity and specificity when predicting evolution in the blastocyst stage (13). Yet in some studies in which injection time is known, other variables coinciding with those of Wong et al. (i.e., synchrony as well as others such as the time when the embryo has five cells [t5; 48.8–56.6 h]) seem to be more important when predicting not only capacity of evolution to the blastocyst stage, but also the implantation capacity of an embryo (12, 26). Recently, another study, performed with a small number of excess ICSI embryos, ruled out EC as a main variable, and tended to accept other variables, such as the time of the second division (from three to four cells) or the time needed to go from five to eight cells, which was significantly shorter in those embryos producing good-quality blastocysts: respectively, 0.7 hours and 5.7 hours versus 5.7 hours and 16.9 hours (27).

CONCLUSION

This multicenter prospective study demonstrates that although the presence of EC is associated with embryo quality in early development stages, its relation with implantation disappears when considering embryo quality at the time of embryo transfer. Moreover, live-birth rates were identical among embryos with and without EC. At the time of embryo transfer, EC would be helpful only when suboptimal-quality embryos are available for transfer. Nevertheless, the implementation of the time-lapse technology will help to analyze in much more detail new aspects of EC that can confirm the lack of clinical relevance of evaluating this parameter for ASEBIR recommendations.

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