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Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability

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A number of non-invasive methods have been proposed to evaluate embryo viability in human in-vitro fertilization programmes. In addition to biochemical analyses, a common method for the selection of embryos prior to transfer involves assessment of embryo quality and morphology. We propose a new method to evaluate embryo viability based on the timing of the first cell division. Fertilized embryos that had cleaved to the 2-cell stage 25 h post-insemination were designated as 'early cleavage' embryos while the others that had not yet reached the 2-cell stage were designated as 'no early cleavage'. In all cases the early cleavage embryos were transferred when available. Early cleavage was observed in 27 (18.9%) of the 143 cycles assessed. There were significantly ($\chi^2 = 4.0$; $P = 0.04$) more clinical pregnancies in the early cleavage group, 9/27 (33.3%), compared with the no early cleavage group, 17/116 (14.7%). No difference was found when comparing key parameters (age, stimulation protocol and semen characteristics) of couples belonging to both groups, pointing to an intrinsic property or factor(s) within the early cleaving embryos. We propose 'early cleavage' as a simple and effective non-invasive method for selection and evaluation of embryos prior to transfer.

Key words: early cleavage/embryo quality/in-vitro fertilization/viability

Introduction

The success rate of human in-vitro fertilization (IVF) remains relatively low when the number of pregnancies is considered in proportion to the number of embryos transferred. The low pregnancy rates per embryo transferred leads to a need to transfer more than one embryo, which in turn increases the chance of multiple pregnancies. To increase pregnancy rates and limit the occurrence of multiple births, a more efficient and rigorous procedure for embryo selection prior to transfer is therefore needed.

Many methods have been suggested to evaluate embryo viability in IVF programmes. A limiting factor is that these measurements need to be non-invasive and not time consuming. Routinely, the embryos selected for transfer are chosen on the basis of their morphology and rate of development in culture. In one such study, Cummins *et al.* (1986) established an embryo quality and embryo development rating and found that good ratings for both result in clinical pregnancies. Other studies have also found advantage in transferring embryos on the basis of a morphological and developmental assessment (Edwards *et al.*, 1984; Hill *et al.*, 1989; Steer *et al.*, 1992). In addition to the above methods, the measurement of several metabolic parameters of the embryos by using non-invasive procedures has also been proposed (Leese, 1987). In one such study Conaghan *et al.* (1993) showed that an inverse relationship existed between pyruvate uptake and human embryo viability. Although these methods are potentially of great benefit they unfortunately require additional expertise and cost, which may be prohibitive in smaller IVF centres.

We therefore propose a new simple method to evaluate embryo viability based on the timing of the first cell division. The earliest time the human zygote can reach the 2-cell stage ranges between 20 and 27 h post-insemination (Trounson *et al.*, 1982; Balakier *et al.*, 1993; Capmany *et al.*, 1996). We have defined a sub-group of embryos that have undergone 'early cleavage'; in that they divide and reach the 2-cell stage 25–26 h post-insemination. We therefore present results examining the relationship between early cleavage of embryos and the incidence of pregnancy. Furthermore, we propose that this may be used as a simple and effective selection criterion for selecting embryos for transfer.

Materials and methods

The study was performed on patients entering the IVF programme of the Clinic of Infertility and Gynaecological Endocrinology, Department of Obstetrics and Gynaecology, University Hospital of Geneva, Geneva, Switzerland between January 1992 and December 1995. In all, 143 cycles were assessed where the patient underwent a routine IVF treatment with transfer. All patients who failed to achieve fertilization or who underwent subzonal microinjection or intracytoplasmic sperm injection (ICSI) were not included. The stimulation protocol adopted by our group has been previously described (Sakkas *et al.*, 1994).

Collected oocytes (day 0) were fertilized using our standard culture medium, Whittingham's T6 (Quinn *et al.*, 1982) supplemented with 10% maternal human serum. Oocyte retrieval took place between 0830 and 1030 hrs. Insemination was performed between 1530 and 1630 (~1600) hrs in tubes. The following morning (day 1) the oocytes were removed from the tubes, washed and placed in 20 μl culture

Table I. The total and mean number (±SD) of oocytes, 2-pronucleate (2PN) oocytes, embryos observed on day 2, embryos transferred, implantation rate and pregnancies according to whether embryos had or had not undergone early cleavage to the 2-cell stage by 25 h post-insemination

Parameter	No early cleavage	Early cleavage	*P*
No. of cycles	116	27	–
No. of oocytes (mean ± SD)	999 (8.7 ± 6.5)	229 (8.5 ± 4.8)	0.91
No. of 2PN oocytes (mean ± SD)	607 (5.23 ± 3.5)	160 (6.07 ± 3.9)	0.28
Early 2-cell embryos (mean ± SD)	0	75 (2.78 ± 2.4)	<0.001
No. of embryos on day 2 (mean ± SD)	511 (4.41 ± 3.2)	156 (5.78 ± 3.9)	0.06
No. of embryos transferred (mean ± SD)	322 (2.78 ± 0.7)	72 (2.67 ± 0.6)	0.47
Implantation rate (%) (fetal heart beat/ embryo transferred)	24/322 (7.5)	17/72 (23.6)	0.0001
No. of clinical pregnancies (%)	17 (14.7)	9 (33.3)	0.045

drops under oil (Light white mineral oil; Sigma Pharmaceuticals, Buchs, Switzerland) in Petri dishes and the presence of two pronuclei assessed. On the same day (day 1), at 1700 hrs (25 h post-insemination), the embryos were examined to see if cleavage to the 2-cell stage had occurred. These embryos were designated as 'early cleavage' embryos while the embryos that had not yet cleaved were designated as 'no early cleavage'. Both groups were maintained in separate culture drops. The following day (day 2) the embryos were transferred between 1000 and 1800 hrs. Routinely, a maximum of three embryos were transferred to the patient. When there were only one or two early cleavage embryos the embryo transfer group was completed by adding the best one or two embryos from the no early cleavage group. In 11 cycles, four embryos were transferred due to advanced maternal age, repeated failure in previous cycles or poor quality embryos. Coincidentally, these patients were all in the no early cleavage group.

On the day of transfer all embryos were assessed for the number of cells per embryo, to ascertain their cleavage rate, and given a quality score based on the presence of fragments and clarity of the cytoplasm of the blastomeres, similar to that previously described by Cummins *et al.* (1986). The ratings given for cell number were: 1 for 1-cell, 2 for 2-cell, 3 for 3-cell, 4 for 4-cell, 6 for between 4–8-cell and 8 for ⩾8-cell embryos. The ratings given to embryos for quality were the same as those used by Cummins *et al.* (1986) except that the values of 0 for poorest and 3 for best embryo were given.

A pregnancy test was conducted 14 days after transfer. Patients that exhibited three consecutive human chorionic gonadotrophin (HCG) values >5 mIU/ml and in which the fetus or fetuses displayed a heart beat by ultrasound examination 4–5 weeks after transfer were considered to have achieved a clinical pregnancy.

The statistical evaluations used were analysis of variance followed by Scheffé's *F*-test for comparison of mean values and χ^2 analysis with continuity correction for comparison of pregnancy rates. Statistical significance was set at $P < 0.05$.

Results

Early cleavage was observed in 27/143 (18.9%) of the cycles. Table I shows the IVF results, including the implantation rate and pregnancy outcome, in patients who had at least one early cleavage embryo compared with those that had none. There was no difference in the number of oocytes, number of 2-pronucleate oocytes or the number of embryos transferred between both groups. Seventy-five early cleaving embryos were observed in 27 cycles. The mean number of embryos per patient on day 2 displayed a tendency to be higher in the early cleavage group compared with the no early cleavage group. Early cleaving embryos implanted at a rate 3-fold higher than no early cleaving embryos. The pregnancy rate in the early cleavage group was double the rate of the no early cleavage group; nine pregnancies in 27 cycles (33.3%) compared with 17 pregnancies in 116 cycles (14.7%) ($\chi^2 = 4.0$; $P = 0.04$). No cases of ectopic pregnancies were observed in either group. The chances of achieving a pregnancy increased along with the number of early cleavage embryos observed per patient (Figure 1). In effect, patients in whom two or more early cleaving embryos were observed had 3-fold the pregnancy rate compared with those with no early cleavage.

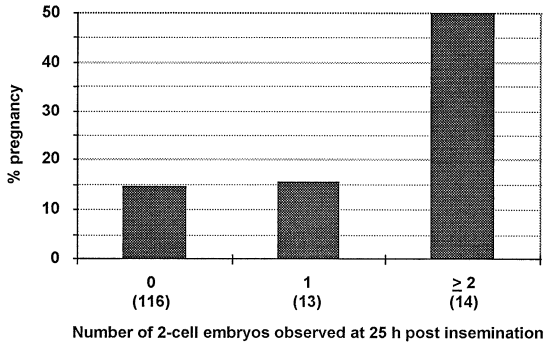

Figure 1. The clinical pregnancy rate in relation to the number of early cleaving 2-cell embryos observed at 25 h post-insemination. The number of cycles for each group is shown in parentheses. ($\chi^2 = 10.6$, DF = 2, $P = 0.005$.)

Table **II.** Female parameters for cycles with and without early cleavage to the 2-cell stage by 25 h post-insemination

Parameter	No early cleavage	Early cleavage	*P*
No. of cycles	116	27	–
Female age (years)	35.1 ± 4.5	34.7 ± 3.9	0.72
Oestradiol concentrations (pg/ml) on:			
Day 0 (day of HCG)	1834.0 ± 1043.4	1952.3 ± 1063.2	0.60
Day 1	2280.1 ± 1348.7	2181.0 ± 1242.5	0.76
Day 2	1139.2 ± 690.2	990.2 ± 621.8	0.43
Ampoules of Pergonal	27.0 ± 12.0	30.0 ± 10.3	0.23

Values are means ± SD.

When a number of key parameters were examined for both groups, we failed to find any differences that influenced the occurrence of early cleavage. The mean age and progress of stimulation, as indicated by oestradiol levels on the day of HCG and oocyte retrieval and the number of ampoules of Pergonal (Serono, Aubonne, Switzerland) used during stimulation, showed no differences between the two groups (Table II). There were also no significant differences concerning the mean age and the semen characteristics of the male partner between both groups (data not shown).

Table III. Clinical pregnancy rates according to the number of embryos transferred in patients who had early and no early cleaving embryos

No. of embryos transferred	No early cleavage (%)	Early cleavage (%)
1	0/8 (0)	0/3 (0)
2	2/21 (9.5)	0/3 (0)
3	14/76 (18.4)	9/21 (42.9)[a]
4	1/11 (9.1)	–

[a]Significantly different ($P = 0.04$) when compared to patients with no early cleavage embryos. These data include mixed transfers as detailed in Table IV.

The clinical pregnancy rate according to the number of embryos transferred is represented in Table III. When three embryos were transferred significantly more pregnancies were obtained in the early cleavage group compared with the no early cleavage group. No comparison was made when one or two embryos were transferred due to the low number of cycles. Table IV shows the implantation rate and the incidence of single, twin and triplet pregnancies when the cohort of the three embryos transferred contained one, two or three early cleavage embryos or all no early cleavage embryos. Interestingly, when all three embryos transferred had undergone early cleavage the pregnancies obtained were multiple.

To examine whether the cohorts of embryos transferred to the patients with and without early cleaving embryos differed, we calculated the mean score of the number of cells and mean quality per embryo. The mean number of cells per embryo and the mean quality of cells per embryo on day 2 for those transferred are presented in Table V. The results show that, while no significant difference was evident in the mean cell numbers of the embryos transferred, the quality rating was significantly higher for the embryos transferred in the patients that had early cleavage embryos. Finally, when a comparison between both groups was made of the pregnancy rates in relation to the number of cells and the quality of embryo transferred, the transfer of embryos of higher cell number and quality in the early cleavage group provided a significantly higher pregnancy rate (Figure 2).

Discussion

The need for a non-invasive method to assess embryo viability has been highlighted previously by Leese (1987). Methods currently available are those that examine biochemical markers such as the measurement of several metabolic parameters, for example: O_2 consumption (Magnusson *et al.*, 1986), pyruvate uptake (Leese *et al.*, 1986; Hardy *et al.*, 1989; Conaghan *et al.*, 1993), glucose uptake and lactate production (Wales *et al.*, 1987; Hardy *et al.*, 1989), the secretion of factors such as platelet activating factor (O'Neill and Saunders, 1984) and the activity of the enzymes involved in fatty acid catabolism, the citrate cycle and the pentose pathway (Chi *et al.*, 1988; Martin *et al.*, 1993). Of these methods the assessment of metabolic criteria appear to be the most effective. For example, analysis of 2–8-cell stage human embryos prior to transfer revealed that pyruvate uptake was significantly lower by embryos that implanted after transfer (Conaghan *et al.*, 1993). More recently, Lane and Gardner (1996) examined glucose uptake and glycolytic activity, using non-invasive quantitative microfluorescence, to prospectively select mouse blastocysts for transfer. They showed that a low glycolytic activity resulted in a 4-fold increase in the pregnancy rate. Although effective, many of these methods need special training and can prove to be time consuming, subsequently limiting their use in routine evaluation of embryo viability.

Currently, many IVF programmes depend on a simple morphological assessment of embryos at the time of transfer. Several systems of embryo grading have been proposed according to the morphology (Hill *et al.*, 1989; Scott *et al.*, 1991), cleavage stage (Edwards, 1984; Cummins *et al.*, 1986; Claman *et al.*, 1987; Bolton *et al.*, 1989) or both (Puissant *et al.*, 1987; Steer *et al.*, 1992). Cummins *et al.* (1986) confirmed the feasibility of such an approach in patients receiving single embryo transfers by showing that combining a subjective score of embryo quality and a rating of cleavage stage was a good predictor of expected pregnancy.

In this study we introduce a novel assessment criterion and show that when a patient has early cleaving 2-cell embryos the chances of achieving a pregnancy are significantly improved. More importantly, we also show that even in patients who had transfers of high quality and better cleaving embryos in the no early cleavage group the pregnancy rates achieved were still higher for the early cleavage group. This raises the question as to why selecting early cleaving embryos appears to be more advantageous than selecting the best embryos at the time of transfer. The timing of the first cell division in humans has been reported by several investigators. Trounson *et al.* (1982) reported that the earliest time the 2-cell stage can be observed was 27 h post-insemination. However, more recently, Balakier *et al.* (1993) reported this time to be at 20–22 h post-insemination while Capmany *et al.* (1996) reported it to be 25 h post-insemination. In fact, Balakier *et al.* (1993) found that 1% of the zygotes reached the 2-cell stage at 20 h post-insemination, 5% at 24 h and 38% by 27 h. These times are in accordance with our observations.

The selection of a critical time-point is essential so as to maximize the differences between embryos. Observations of embryo development in culture are sometimes made infrequently (commonly at 16–18 h post-insemination to check for pronuclei and again at ~40 h just before transfer) so that precise data on cleavage timing is usually not available. Bavister (1995) highlighted this problem by stating that the examination of embryos at arbitrary time points during development can be quite misleading with respect to categorizing the stage of development reached and 'timeliness' of development. This problem is illustrated in Figure 3. In two groups of embryos (early and no early cleavage) observation of embryos at point A shows no difference between embryos of the same cell stage (2-cell stage), although in the early cleavage group the embryo has divided before the no early cleavage embryo. The same applies to point B for the selection of embryos for transfer. In both groups the embryos are at the 4-cell stage and are of the same stage despite one having cleaved hours before (in the early cleavage group) while the other has just

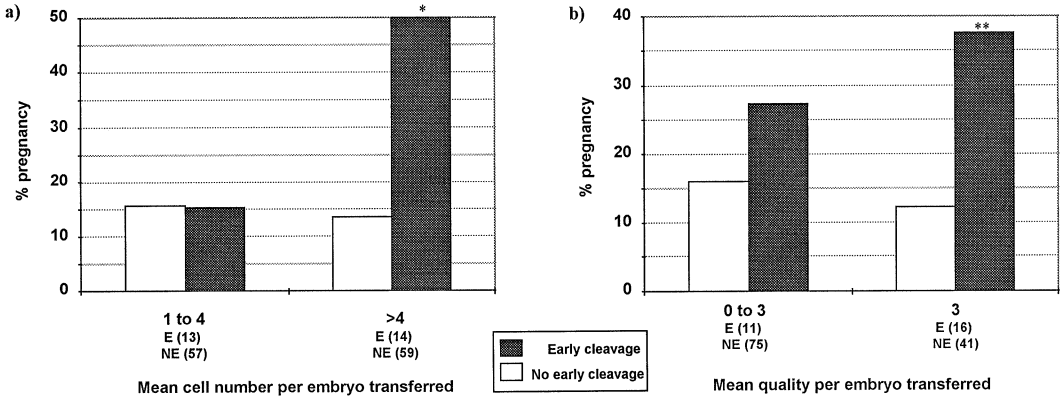

Figure 2. The clinical pregnancy rate in relation to (**a**) the mean cell number per embryo transferred and (**b**) the mean quality per embryo transferred for the early cleavage (E) and no early cleavage (NE) groups. The number of cycles for each group is shown in parentheses. *Significant difference ($\chi^2 = 7.1$, $P = 0.008$) in pregnancies when compared to the no early cleavage group. **Increased but no significant difference ($\chi^2 = 3.3$, $P = 0.07$) in pregnancies when compared to the no early cleavage group.

Table IV. Pregnancy in relation to the number of early and no early cleavage embryos transferred in patients receiving three embryos at transfer

No. of embryos transferred		Pregnancy				
No early cleavage	Early cleavage	Single	Twins	Triplets	Implantation rate[a] (%)	Total[b] (%)
Patients with no early cleaving embryos						
3	0	10	2	2	20/228 (8.8)	14/76 (18.4)
Patients with early cleaving embryos						
2	1	1	1	0	3/9 (33.3)	2/3 (66.6)
1	2	1	1	0	3/18 (16.7)	2/6 (33.3)
0	3	0	4	1	11/36 (30.6)	5/12 (41.7)

[a]Number of fetal heart beats per embryo transferred.
[b]Number of clinical pregnancies per cycle.

Table V. Mean number of cells per embryo and mean quality per embryo on day 2 of embryos transferred in patients with and without early cleavage to the 2-cell stage by 25 h post-insemination

	Embryos of no early cleavage patients: No early cleavage	Embryos of early cleavage patients		
		All embryos	No early cleavage	Early cleavage
No. of embryos transferred	322	72	21	51
No. of cells[a]	4.3 ± 1.7	4.5 ± 1.2	4.2 ± 1.7	4.8 ± 1.9
Quality of cells[a]	2.3 ± 0.8[b,c]	2.6 ± 0.6[b]	2.6 ± 0.5	2.7 ± 0.5[c]

[a]Means ± SD.
[b,c]Same letters differ significantly: [b]$P = 0.04$ and [c]$P = 0.02$.

cleaved (the no early cleaved group). Although in a group of embryos there will be those present that have a higher cell number, the embryos at the same stage cannot be distinguished. For example, if a patient had one 8-cell and six 4-cell embryos of similar quality, the 8-cell embryo is selected but a problem arises as to which 4-cell embryo is more advanced. Examination of early cleavage therefore gives more discrimination between groups.

An analysis of the patient groups giving rise to early cleavage and no early cleavage showed no differences in the number of oocytes, the rate of fertilization, nor when examining for parameters associated with males and females. The fact that we could not find any differences in the characteristics of the female partners as regards to the age, type of infertility (data not shown), oestradiol concentrations and stimulation protocol excludes possible female factors such as endometrial receptivity as the cause of the differences in pregnancy rates between both groups. As no ascertainable difference was evident in the male and female characteristics, this points to the possibility of an enhanced intrinsic oocyte or embryo quality in the cases where early cleavage is observed. The finding of a higher number of embryos on day 2 in the early cleavage group, although not significant, also indicates that a better embryo development in the early cleavage group may indeed be present. One possibility is that the early cleavage could arise due to time differences in fertilization and sub-

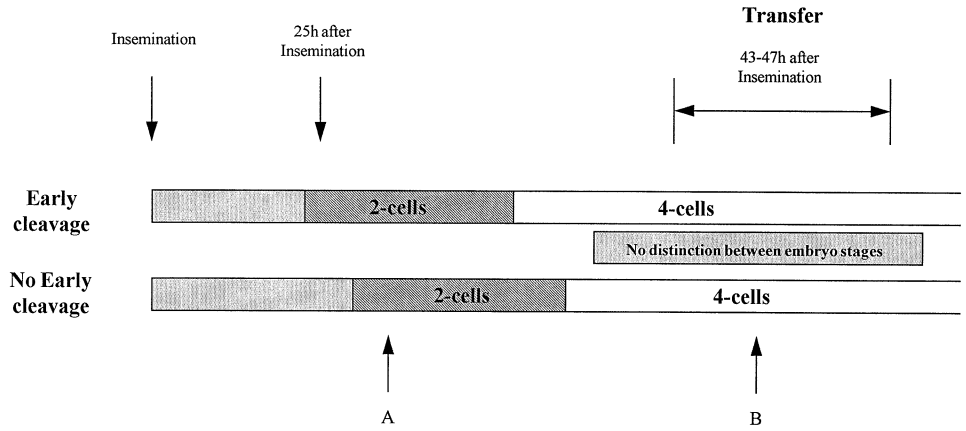

Figure 3. Importance of specific time points for distinguishing embryo cleavage during screening (see Discussion for details).

sequent events simply due to the difference in the time required for sperm penetration through the cumulus cells and zona pellucida. The latter depends on the maturity of the oocytes, as less mature oocytes are usually fertilized later than the mature ones, leading to a delayed cleavage (Balakier *et al.*, 1993). Contrary to this hypothesis, however, are similar results we have obtained when separating early cleavage and no early cleavage 2-cell embryos after ICSI. In this case the time of sperm entry and the maturity of the oocytes is regulated and an even more marked significant difference is evident in pregnancy rates between the two groups (unpublished data). These data would exclude a possible influence of the time of fertilization and oocyte maturity on early cleavage.

Whereas early cleavage could be due to a selection of the oocytes that were primed to fertilize, a further explanation of the higher viability of the early cleavage embryos could also arise due to the presence of an unknown factor(s) within the oocyte which favours or promotes the early division of embryos after fertilization. The intrinsic nature of a certain population of embryos to cleave faster has previously been observed in mouse embryos. Mouse embryos have been shown to possess a gene, associated with the major histocompatibility complex, which manifests itself as two functional alleles. This gene has been called the preimplantation embryo development (*Ped*) gene. Mouse embryos homozygous for the dominant fast *Ped* allele cleave at a faster rate than those which are heterozygous, while those homozygous for the slow *Ped* allele cleave at the slowest rate (Goldbard and Warner, 1982). It has been shown that the *Ped* phenotype is an intrinsic property of the embryo independent of the uterine environment (Brownell and Warner, 1988). In addition, there is no effect of the *Ped* gene on time of ovulation or fertilization as it initially acts at the time of the first cleavage division. The *Ped* phenotype has been linked with production of the Qa-2 antigen. The way in which the presence of the Qa-2 antigen on the cell surface induces the embryo to divide at an earlier and faster rate is unknown. It is likely that the mouse *Ped* gene has a human homology within human leukocyte antigen (HLA)-F (Stroynowski, 1990). More recently, Jurisicova *et al.* (1996) have demonstrated the expression of HLA-G throughout preimplantation development in the human, and proposed that it could be a functional homologue to the mouse Qa-2 antigen. Most importantly, the same authors demonstrated a correlation of HLA-G mRNA expression with improved cleavage rate in human preimplantation embryos.

In this study we have shown that the evaluation of early cleaving 2-cell embryos in an IVF programme could be an effective and valuable method of assessment of embryo viability, in that it provides a strong prognostic indication of the likelihood of pregnancy. The verification of this observation in a larger study and by independent groups is eagerly awaited. The cause of early cleavage in embryos is not obvious but indications exist that it could be an intrinsic factor within the oocyte or embryo. An inherent genetic control of such early cleavage could be postulated. The search for such a factor may be important in understanding the timing of events during the first cell cycle in the human and could possibly provide an indicator for pregnancy in a given cycle.

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