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Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos *in vitro*

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BACKGROUND: The absence of reliable markers for the identification of viable embryos for transfer at the early cleavage stage is likely to contribute to the generally low implantation rates and high incidence of multiple gestation in IVF treatment. In this study, we investigate the relationship between timing of first cleavage and the incidence of blastocyst formation *in vitro*. METHODS: Couples ($n = 70$) with at least one embryo remaining after transfer were included in the analyses. All embryos ($n = 579$) were examined for early cleavage at 25 h after insemination. Following embryo transfer, the remaining embryos ($n = 426$) were cultured until day 7 of development, and assessed for blastocyst formation. RESULTS: Eighty-five embryos (14.7%) cleaved to the 2-cell stage within 25 h of insemination; 26 of these were selected for transfer on day 2. Of the 59 embryos remaining in culture, 19 (32.2%) developed to the blastocyst stage; this was a significantly higher number than was observed in embryos (61/367; 16.6%) that failed to cleave within 25 h of insemination ($P < 0.01$). Within these two groups of embryos the proportion of hatched blastocysts was 11/59 (18.6%) and 26/367 (7.1%) respectively ($P < 0.005$). CONCLUSIONS: These findings indicate that early cleavage is indicative of increased developmental potential in human embryos and may be useful as an additional criterion in the selection of embryos for transfer.

Key words: blastocyst formation/early cleavage/first cell cycle/human embryos

Introduction

The ability to identify viable embryos is critical to the success of IVF treatment. The predominant practice in clinical IVF is to select embryos for transfer based on an assessment of cell number and morphological appearance at the time of transfer on day 2 or 3 of development. This has been shown to be positively correlated with implantation and pregnancy (Cummins *et al*., 1986; Puissant *et al*., 1987; Staessen *et al*., 1992; Giorgetti *et al*., 1995; Ziebe *et al*., 1997). However, with the development of aggressive ovarian stimulation protocols and improved culture conditions for early embryos, individual patients may produce multiple good quality embryos, with equivalent cell numbers and morphological scores. Taken together with the increasing pressure to reduce the number of embryos replaced in the uterus, due to the high incidence of multiple pregnancy (Coetsier and Dhont, 1998; Gerris and Van Royen, 2000), these developments conspire to make the selection of embryos for transfer an increasingly important and difficult task.

A number of strategies, ranging from pronucleate stage selection to blastocyst transfer, have been devised to help improve the prediction of embryo viability. Studies in which pregnancy and implantation rates were compared following transfer of blastocyst or cleavage stage embryos have produced conflicting results. While some studies have reported that transfer of blastocysts results in higher implantation rates than transfer of cleavage stage embryos (Gardner *et al*., 1998; Marek *et al*., 1999; Schoolcraft *et al*., 1999; Milki *et al*., 2000), other studies have found no difference (Coskun *et al*., 2000; Huisman *et al*., 2000). However, the practice of blastocyst transfer is not in widespread use, partly because of a general lack of experience in prolonged embryo culture, as well as anxieties about those patients whose embryos arrest before blastocyst formation (Van Blerkom, 1997). Furthermore, an increased incidence of monozygotic twinning after blastocyst transfer has been reported (Behr *et al*., 2000; da Costa *et al*., 2001).

An alternative strategy, which has evolved in situations where culture beyond the zygote stage is not compatible with religious beliefs or legal requirements, is to select embryos on the basis of pronucleate stage morphology. The evidence indicates that a grading system based on morphological charac-

teristics, including the extent of pronuclear apposition and nucleolar alignment, is predictive of implantation potential (Scott and Smith, 1998; Tesarik and Greco, 1999; Ludwig *et al*., 2000) and blastocyst formation (Scott *et al*., 2000). Consistent with this, a recent study, encompassing experimental evidence and mathematical modelling, supports the idea that the viability of human embryos is already determined at the 1-cell stage (Hardy *et al*., 2001).

The 1-cell stage of development represents the return to mitotic division following completion of maternal meiosis. In the first cell cycle, S phase, in which the chromosomes replicate, and M phase, in which the replicated chromosomes segregate, are separated by two gap phases G1 and G2. Entry into G1 is marked by the appearance of pronuclei, which persist until the transition from G2 into M phase. Observations of human zygotes indicate variability in the timing of all cell cycle transitions following sperm entry (Balakier *et al*., 1993; Capmany *et al*., 1996; Payne *et al*, 1997; Nagy *et al*., 1998). The culmination of this is that the onset of first cleavage (exit from M phase) is observed over a time-span of >8 h (between 22 and >30 h after sperm entry) (Payne *et al.*, 1997; Nagy *et al.*, 1998).

Studies in other species have shown a relationship between the timing of completion of the first cell cycle and subsequent developmental potential. In mice and cattle, early onset of first cleavage is associated with increased blastocyst formation and implantation (McLaren and Bowman, 1973; Grisart *et al*., 1994; Lonergan *et al*., 1999). The suggestion that such an association might also exist in human embryos comes from the finding that patients who produced early cleaving (EC) embryos had higher pregnancy and implantation rates than those who did not (Shoukir *et al*., 1997; Sakkas *et al*., 1998). However, an unequivocal correlation between time from insemination to first cleavage and embryo development potential has not yet been established, since the studies to date (Shoukir *et al*., 1997; Sakkas *et al*., 1998) have preferentially replaced EC embryos when available. Any differences in the implantation potential of EC and non-early cleaving (NEC) embryos in the population of patients generating both embryo types were therefore not examined. Also, it is possible that other aspects of fecundity, such as improved endometrial receptivity, may account for the improved treatment success of patients generating EC embryos compared with patients with only NEC embryos.

Given its ease of application and non-subjective nature, we were interested to explore further the usefulness of early cleaving as a marker of embryonic viability. Here, we report the findings of a study in which we compared blastocyst formation *in vitro* of embryos that cleaved within 25 h of insemination with those that cleaved later. Because the early cleaving status of each embryo was withheld until after completion of the embryo transfer, we were able to evaluate whether there was a natural bias in favour of selecting EC embryos for transfer based on morphological criteria on day 2 of development.

Materials and methods

Source of embryos

The study was performed on embryos produced by 70 sequential couples undergoing IVF treatment, with at least one embryo remaining after embryo transfer. The culture of embryos remaining after transfer until day 7 after fertilization to monitor blastocyst development was part of routine treatment at the time of the study (January–June 1998) and no ethical approval was required.

Treatment regime

Ovulation was induced using a standard protocol of GnRH analogue (Suprefact; Hoechst, Hounslow, UK) and FSH (Metrodin HP or Gonal F; Serono, London, UK) at a daily dose of 150–300 IU for 10–15 days, followed by 5000 or 10 000 IU HCG, (Profasi; Serono). Follicles were aspirated 38–40 h after HCG administration, using ultrasound guidance. Retrieved oocytes were transferred to individual 100 µl droplets of oocyte culture medium (OCM) under mineral oil (Sigma, Poole, Dorset, UK). OCM consisted of Earle's balanced salt solution (EBSS; Life Technologies, Paisley, UK) supplemented with 25 mmol/l sodium bicarbonate (Sigma Hybri-max), 0.5 mmol/l sodium pyruvate (tissue culture grade; Sigma, UK), 10% (v/v) of a 4.5% solution of human serum albumin (HSA; Immuno Ltd, Sevenoaks, Kent, UK), 10 µg/ml gentamycin sulphate (ICN; Thame, Oxfordshire, UK) and 100 IU/ml benzylpenicillin sodium (Crystapen; Brittania Pharmaceuticals, Redhill, Surrey, UK).

Sperm for insemination were isolated by centrifugation at 200 *g* on a discontinuous density gradient composed of 90 and 45% Percoll solutions in HEPES-buffered EBSS (Percoll; Pharmacia, Sweden; HEPES; Sigma). Between 25 000 and 50 000 sperm were added to each oocyte at 41–43 h after HCG administration (day 0).

At 18–20 h post-insemination (day 1) the oocytes were mechanically denuded of their cumulus cells and those showing two pronuclei were transferred to individual 100 µl droplets of embryo culture medium (ECM) under mineral oil. ECM comprised EBSS supplemented with 15% (v/v) of a 4.5% solution of human serum albumin (HSA) and 0.5 mmol/l sodium pyruvate. All embryos were examined at 24.5–25.5 h post-insemination and those which had divided to become two cells were termed early cleaving (EC) embryos and those which had not yet divided were termed non-early cleaving (NEC) embryos.

On day 2, at 42–48 h post-insemination, each embryo was assessed for cell number and assigned a morphology score between 0 and 1.0. The latter value was a semi-quantitative assessment of embryo quality, taking into account the degree of fragmentation, uniformity of blastomere size and cytoplasmic appearance. Up to three of the fastest dividing and highest scoring embryos were selected for transfer. The timing of first cleavage of the embryos was unknown to the operator at the time of transfer, and therefore did not influence the selection process.

Blastocyst culture

Following transfer of the best quality embryos, EC and NEC embryos from individual patients were pooled and cultured in separate wells of a 4-well dish (Nunclon, Life Technologies, Paisley, UK). The number of embryos cultured in each well ranged from 1–7 (mean = 2.3) and 1–13 (mean = 3.3) for EC and NEC embryos respectively. Each well contained 0.75 ml of Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1), supplemented with 2% Ultroser G (all from Life Technologies), overlaid with 0.25 ml oil. Embryos were inspected on day 7 for development to the blastocyst stage. Only expanded, hatching or hatched blastocysts were recorded. All incubations were performed at 37°C in a humidified environment of 5% CO_2 in air.

Statistical analysis

Statistical evaluations used were contingency table (χ^2) analyses for comparison of proportional values and two-sample Student's *t*-test for comparison of mean values.

Table I. Comparison of IVF treatment cycle parameters for group A patients producing both early cleaving (EC) and non-early cleaving (NEC) embryos and group B patients producing NEC embryos only

Parameter	Group A	Group B	*P*
No. of cycles	32	38	–
Mean female age (years) ± SD	32.2 ± 4.0	32.8 ± 3.5	NS
Stimulation (mean ampoules ± SD)	39.4 ± 9.2	39.6 ± 7.3	NS
Mean follicles ± SD	15.97 ± 5.78	15.21 ± 7.32	NS
Mean oocytes ± SD	11.16 ± 4.21	11.58 ± 5.36	NS
Mean 2PN embryos ± SD	8.72 ± 3.59	7.89 ± 3.60	NS
Clinical pregnancies (%)	10/32 (31.3)	4/38 (10.5)	0.05
Implantation rate (%)	15/70 (21.4)	5/83 (6.0)	0.005

PN = pronuclei; NS = not significant.

Table II. Proportion of early cleaving (EC) and non-early cleaving (NEC) embryos developing to expanded or hatching blastocyst stage following extended culture to day 7

	EC	NEC
Embryos cultured	59	367
Blastocyst (%)	19 (32.2)[a]	61 (16.6)[a]
Hatched (%)	11 (18.6)[b]	26 (7.1)[b]

Same letters indicate significant differences, χ^2-test: [a]$P < 0.01$; [b]$P < 0.005$.

Table III. Proportion of early cleaving (EC) and non-early cleaving (NEC) embryos developing to expanded or hatching blastocyst stage following extended culture to day 7 in relation to patient group

	Group A		Group B
	EC	NEC	NEC
Total no. of blastocysts (%)	19/59 (32.2)[a,b]	27/150 (18.0)[b]	34/217 (15.7)[a]
Hatching rate (%)	11/59 (18.6)[c]	14/150 (9.3)	12/217 (5.5)[c]

Same letters indicate significant differences, χ^2-test: [a]$P < 0.01$; [b]$P < 0.05$; [c]$P < 0.001$.

Results

Incidence of early cleaving in human embryos

A total of 579 zygotes from 70 couples were assessed for cleavage at 25 h after insemination. Of these, 85 (14.7%) were at the 2-cell stage (EC), and 494 (85.3%) were still at the 1-cell stage (NEC). Thirty-two couples (group A, 46% of patients) produced a mix of EC and NEC embryos while 38 couples (group B) produced only NEC embryos. There was no difference between the two patient groups in female age, ovarian response or fertilization rate (Table I). However, group A patients had a significantly higher incidence of pregnancy (31.3 versus 10.5%; $P < 0.05$) and implantation (21.4 versus 6%; $P < 0.005$) than group B patients. This is consistent with findings in previous studies (Shoukir *et al*., 1997; Sakkas *et al*., 1998), but as with these studies, our implantation data were insufficient to distinguish between embryonic viability and other aspects of fecundity. However, we were able to examine the relationship between embryonic developmental potential and timing of first cleavage by assessing blastocyst development of EC and NEC embryos during culture *in vitro*.

Early cleaving embryos have a greater potential for blastocyst formation

Embryos remaining ($n = 426$) after transfer were cultured until day 7; 59 of these were EC and 367 were NEC embryos. The proportion of EC embryos that developed to the blastocyst stage by day 7 was 19/59 (32.2%); this was significantly higher ($P < 0.01$) than in NEC embryos (61/367; 16.6%). The proportions of hatched blastocysts were 18.6 and 7.1% for EC and NEC embryos respectively ($P < 0.005$; Table II). Interestingly, the proportions of hatched blastocysts corresponded closely with the implantation rates for the two patient groups.

We next asked whether the increased blastocyst formation of EC embryos was truly a function of the timing of first cleavage or merely a reflection of a generally superior developmental potential among cohorts of embryos produced by group A patients. We therefore analysed the incidence of blastocyst formation and hatching of EC and NEC embryos, according to whether they were obtained from group A or group B patients (Table III). Within group A patients, the incidence of blastocyst formation was significantly higher ($P < 0.05$) for EC than for NEC embryos, and the NEC embryos of group A patients showed a similar incidence of blastocyst formation to those of group B patients. The proportions of EC and NEC embryos from group A patients developing to the hatched blastocyst stage were 18.6 and 9.3% respectively (borderline significance: $P = 0.06$). While the proportion of NEC embryos from group B patients developing to hatched blastocyst stage was 5.5%, this was significantly lower than that of EC embryos ($P < 0.001$) but not NEC embryos from group B patients (Table III).

Early cleaving is associated with higher mean cell number and better morphology on day 2

To investigate whether the greater potential for blastocyst formation of EC embryos was evident at early stages of development, we compared the grades assigned on day 2 with the EC and NEC embryos that were subsequently cultured until day 7, i.e. those embryos not selected for transfer. EC embryos ($n = 59$) had significantly higher mean cell numbers

Table IV. Mean cell number and morphology score on day 2 of early cleaving (EC) and non-early cleaving (NEC) embryos cultured to the blastocyst stage (day 7)

	Group A		Group B
	EC	NEC	NEC
No. of embryos	59	150	217
Cell number	3.98 ± 0.29[a,b]	3.64 ± 0.99[a,c]	3.14 ± 1.10[b,c]
Morphology score	0.85 ± 0.11[d,e]	0.80 ± 0.15[d]	0.81 ± 0.15[e]

Results are expressed as means ± SD.
Same letters indicate significant differences, two-sample *t*-test:
[a,b,c]$P < 0.001$; [d,e]$P < 0.01$.

Table V. Proportions of early cleaving (EC) and non-early cleaving (NEC) embryos available for transfer and selected for transfer to patients producing a mix of both embryo types (group A patients)

	EC	NEC
Available embryos ($n = 279$)	85 (30.5)	194 (69.5)
Transferred embryos ($n = 70$)	26 (37.1)	44 (62.9)
P-value	NS	NS

Values in parentheses are percentages.
NS = not significant.

($P < 0.001$) and mean morphology scores ($P < 0.01$) than NEC embryos ($n = 367$). The improved quality of EC embryos was irrespective of whether the NEC embryos were derived from group A or group B patients, although NEC embryos from group A patients had a significantly higher mean cell number than NEC embryos from group B patients (Table IV).

There is no bias in favour of selection of early cleaving embryos for transfer

The study protocol dictated that selection of embryos for transfer was blind with respect to the timing of first cleavage. On this basis, 5/32 patients in group A had only EC embryos replaced, 14/32 had a mixture of EC and NEC replaced, and 13/32 had only NEC embryos replaced. Table V shows that for group A patients, the prevalence of EC embryos in the cohort of embryos available for transfer ($n = 279$) and in the cohort of embryos transferred ($n = 70$) was not significantly different, indicating that there was no bias in the selection process. In accordance with this, analysis of mean cell number and morphology score showed that among the population of embryos selected for transfer, there was no difference between EC and NEC embryos (Table VI).

Table VI. Mean cell number and morphology score on day 2 of early cleaving (EC) and non-early cleaving (NEC) embryos selected for transfer to group A patients

	EC	NEC	*P*-value
No. transferred	26	44	–
Cell number	4.27 ± 0.72	4.16 ± 0.83	NS
Morphology score	0.92 ± 0.07	0.89 ± 0.09	NS

Results are expressed as mean ± SD.
NS = not significant.

Discussion

In agreement with the findings of others (Shoukir *et al*, 1997), the data presented here show that couples who produced embryos that cleaved within 25 h of insemination yielded higher pregnancy and implantation rates than those who did not. A correlation between early cleavage and implantation was not established in previous studies (Shoukir *et al*., 1997; Sakkas *et al*., 1998) for two main reasons. First, the number of cases in which only EC embryos were replaced was small; second, the preferential replacement of EC embryos, when available, prevented any possible comparison of the implantation potential of EC and NEC embryos of equivalent quality on day 2. By relating the timing of first cleavage to blastocyst formation and hatching, we have established that the developmental competence of EC embryos *in vitro* was significantly higher than their NEC counterparts. Importantly, this difference was irrespective of whether the NEC embryos were from patients producing a mix of EC and NEC embryos or NEC embryos only. The improved development potential of EC embryos *in vitro* was therefore a function of the timing of first cleavage rather than a 'patient specific effect'.

Our results also indicate that there is no natural bias in favour of selecting EC embryos for transfer. Consistent with this, we found that the best quality NEC embryos, i.e. those selected for transfer, were morphologically indistinguishable from the best quality EC embryos. However, this was not the case among the embryos that were not selected for transfer. Within this population, EC embryos had significantly higher cell numbers and morphology scores than NEC embryos regardless of whether they originated from group A or group B patients. Although NEC embryos produced by group A patients had significantly more cells than those of NEC embryos produced by group B patients, this did not lead to improved blastocyst formation.

A positive correlation between early onset of cleavage and blastocyst formation has also been reported in mice (McLaren and Bowman, 1973) and bovine embryos (Grisart *et al*., 1994; Lonergan *et al*., 1999). In these studies, the blastocysts with early cleavage were found to have more cells than their later cleaving counterparts (McLaren and Bowman, 1973; Lonergan *et al*., 1999). This was found to be attributable to the differences in the timing of first cleavage rather than to differences in the rate of progression of subsequent cell cycles (McLaren and Bowman, 1973).

How might the timing of first cleavage be linked to blastocyst formation? The transition from fertilized oocyte to 2-cell embryo relies upon a highly regulated sequence of cell cycle events, which are initiated by sperm-induced, repetitive transient increases in oocyte free calcium concentration (Kline and Kline, 1992). There is evidence from studies in which mammalian oocytes were activated by pulsatile electrical stimulation that the dynamics of these calcium signals influence (i) the time course of pronuclear formation (Vitullo and Ozil, 1992), which marks entry into G1 of the first cell cycle, (ii)

the ability to undergo blastocyst formation (Ozil, 1990), and (iii) implantation (Ozil and Huneau, 2001). It has also been reported that the G2/M transition in the first cell cycle of mouse zygotes is dependent upon a calcium-releasing activity acquired by the pronuclei during fertilization or activation (Kono *et al*., 1996). Thus, it could be hypothesized that asynchrony between zygotes in the timing of first cleavage and variability in their capacity to undergo blastocyst formation may be due to differences in the ability of individual sperm to stimulate calcium transients, and/or differences in the ability of oocytes to respond to that stimulus. The finding that oocytes acquire the ability to undergo repetitive calcium transients during their maturation process (Carroll *et al.*, 1994; Herbert *et al*., 1997) suggests that oocyte maturity may be an important determinant of the timing of first cleavage and subsequent developmental potential.

A possible alternative or additional mechanistic link between timing of first cleavage and blastocyst formation lies in the fidelity of DNA replication. The duration of S phase of the first cell cycle has been shown to influence blastocyst formation. A longer S phase in association with a shorter G1 in the case of bovine embryos (Comizzoli *et al*., 2000), or shorter G2 in the case of mouse embryos (Schabronath and Gartner, 1988), gives rise to improved blastocyst formation. The duration of S phase is paternally regulated (Schabronath and Gartner, 1988; Comizzoli *et al*., 2000). However, regulation of the timing of entry into S phase appears to differ between species, being regulated by maternal factors in mice (Schabronath and Gartner, 1988) and paternal factors in cattle, in a manner that, in hamsters at least, is not dependent upon sperm nuclear decondensation (Naish *et al*., 1987). It is conceivable that zygotes with shorter S phases are predisposed to incomplete or aberrant DNA replication. This is unlikely to be compatible with normal development to the blastocyst stage and could impose a delay in progression through G2 and M phase of the first cell cycle by activating a DNA structure checkpoint (Nigg, 2001), such as has been identified in mouse zygotes (Fulka *et al*., 1999).

In conclusion, our analyses show that in humans, as in other species (McLaren and Bowman, 1973; Grisart *et al*., 1994; Lonergan *et al*., 1999), early onset of first cleavage is associated with increased blastocyst formation. Given its ease of application and lack of scope for subjectivity, early cleaving could potentially be used as an additional marker of viability when selecting embryos for transfer. This would be especially useful in cases where numerous good quality embryos are produced and/or the risk of multiple pregnancy is increased. However, it will be important to establish whether the increased blastocyst formation of EC embryos equates to increased implantation potential. While blastocyst formation represents an important milestone in embryonic development, it is not necessarily synonymous with viability (Van Blerkom, 1997). Although our data suggest that ability to develop to the hatched blastocyst stage may correlate with implantation potential, an unequivocal correlation between implantation potential and timing of first cleavage awaits collection and analysis of sufficient homologous data from group A patients.

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