Article

Early embryo development is an indicator of implantation potential

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Abstract

To maximize the chances of pregnancy during assisted reproduction treatment, it is important to be able to identify embryos with high implantation potential. Embryos which divide more quickly following insemination have been shown to produce higher pregnancy and implantation rates than those which divide later. The aim of this study was to compare the developmental potential of early cleaving embryos with those in which the pronuclear membranes had broken down at the time of scoring. Normally fertilized zygotes (n = 2447) were assessed 25–27 h post-insemination and categorized according to developmental stage (pronuclei visible, no pronuclei, or early cleavage to two cells). Pregnancy and implantation rates were assessed in cycles where embryos selected for transfer were at an equivalent stage 25–27 h post-insemination. A significantly higher implantation rate was achieved following transfer of either early cleavage embryos or those which had no pronuclei compared with embryos with intact pronuclei when assessed 25–27 h post-insemination/microinjection. The correlation between early cleavage and an improved pregnancy and implantation rate was confirmed. Scoring for the presence of early cleavage or status of pronuclei is quick and objective and provides information that may be used to discriminate between morphologically equivalent embryos at a later stage in development.

Keywords: *early cleavage, implantation, pronuclear membrane breakdown*

Introduction

One of the major challenges in clinical assisted reproduction has been the ability to select accurately those embryos from a cohort that are most likely to implant and give rise to a clinical pregnancy. Historically, three or more embryos were often transferred to increase the likelihood of pregnancy. This procedure led to the establishment of multiple gestations in many cases, with increased health risks for both mother and offspring. More recently, a greater understanding of embryo physiology has led to improvements in culture conditions (Gardner, 1998) with concomitant increase in embryo viability, thus increasing further the chances of multiple pregnancy following transfer of several embryos. There is therefore increasing pressure to replace fewer embryos, with directives in several countries, including the Human Fertilisation and Embryology Authority in the UK, to replace no more than two embryos, with the eventual aim being to move towards single embryo transfers as routine practice (Gerris *et al*., 2002; Hamberger and Hazekamp, 2002). Indeed, some units are now transferring single embryos as standard practise with no obvious reduction in pregnancy rates (De Sutter *et al*., 2003). To ensure the best chance of a pregnancy following transfer of only one embryo, it is essential that embryologists maximize their ability to identify embryos with the highest implantation potential from within a larger cohort, using all the information available to them. Despite the development of sophisticated techniques to try and refine embryo selection procedures (reviewed in Boiso *et al*., 2002), including assessment of embryo metabolism (Johnson and Everitt, 2000; Jones *et al*., 2001; Houghton *et al*., 2002) and identification of euploid embryos by PGD-AS (reviewed in Munné, 2003; Munné *et al*., 2003), embryo morphology has routinely been the major tool used for selecting the highest quality embryos for transfer. In many centres, selecting embryos on the basis of morphology is likely to remain the most common method used for the majority of routine assisted reproduction cycles.

A number of morphological criteria, applicable at various times during early development, have been used to rank embryos in order of quality. Edwards *et al*. (1984) were the first to recognize that faster cleaving embryos were more likely to give rise to a pregnancy and since then, developmental stage and morphological appearance at the time of transfer have been used as the most compelling indication of quality, based on the results of many studies showing that these factors are positively related to implantation and pregnancy rates (Cummins *et al*., 1986; Steer *et al*., 1992; Ziebe *et al*., 1997). More recently, observations at earlier stages of development have been proposed as additional non-invasive markers of embryo viability. In several studies, the size of both pronuclei and the pattern of nucleoli within each pronucleus has been related to embryo implantation potential (Scott, 2003; Scott and Smith, 1998; Tesarik and Greco, 1999; Scott *et al*., 2000). This embryo screening method has been particularly important in Germany, where German embryo protection laws allow a maximum of only three zygotes to be cultured for embryo transfer, while the rest must be cryopreserved at the pronucleate stage of development for future use (Zollner *et al*., 2002). A further indication that visual cues present in the first 24 h of development could be used to enhance embryo selection procedures was reported in 1997, when the first paper was published which indicated that timing of the first cleavage division could predict embryo competence and pregnancy outcome (Shoukir *et al*., 1997). Since then, several other groups have reported similar findings (Sakkas *et al*., 1998; Bos-Mikich *et al*., 2001; Lundin *et al*., 2001; Lundqvist *et al*., 2001; Fenwick *et al*., 2002).

The association between the timing of completion of the first cell cycle and developmental potential is not a new one; studies in other species as early as 1973 have pointed towards such a relationship. McLaren and Bowman were the first to demonstrate this in mouse embryos in 1973 (McLaren and Bowman, 1973) and increased blastocyst formation was associated with early first cleavage in this and other studies in bovine embryos (Grisart *et al*., 1994; Lonergan *et al*., 1999). More recently, an unequivocal relationship between the time taken from insemination to first cleavage and pregnancy outcome in humans has been shown (Lundin *et al*., 2001). Early cleavage of human embryos at 25–27 h post-microinjection was an independent predictor of birth following intracytoplasmic sperm injection (ICSI) treatment, but not conventional IVF (Lundin *et al*., 2001). Furthermore, a significant relationship has been shown between the implantation rate (IPR) and blastocyst formation of embryos from patients with early cleaving embryos (Fenwick *et al*., 2002).

On the basis of these and other studies and since it represents a straightforward, non-subjective and non-invasive method of assessing embryo quality, scoring of embryos for early cleavage at 25–27 h post-insemination/microinjection was introduced as a routine procedure in the Guy's Hospital unit some 4 years ago. It was observed that, in addition to embryos which had completed their first cleavage at the time of scoring and those which still had visible pronuclei, a further category of embryos existed in which the pronuclear membranes had broken down, the embryo presumably preparing to divide, at the time of assessment. The aim of this study was to examine whether early cleavage at 25–27 h post-insemination /microinjection was a significant predictor of treatment outcome and to assess whether embryos with no pronuclei when scored at this time also demonstrated an increased developmental competence compared with those zygotes with pronuclei still intact.

Materials and methods

Source of embryos

The study was conducted between August 1999 and September 2001 at the Assisted Conception Unit, Guy's and St Thomas' Hospitals, London, UK. Data were used from 349 couples who underwent 352 treatment cycles. All normally fertilized zygotes that were morphologically assessed 25–27 h after insemination/microinjection were included in the study, but only patients who had embryo transfer on day 2 (46–50 h post-insemination/microinjection) were included in the pregnancy data.

Treatment regime

The protocol for ovarian stimulation remained unchanged during the study period. Patients underwent pituitary down-regulation using buserelin (Suprefact; Hoechst UK Ltd, Hounslow, Middlesex, UK) in a mid-luteal start long protocol. Gonadotrophin treatment was initiated following satisfactory pituitary suppression as evidenced by a thin endometrium and absence of follicular activity or ovarian cysts. Ovarian stimulation was achieved using a daily FSH dose of 150–450 IU of highly purified urinary FSH (Metrodin HP; Serono Laboratories Ltd, Welwyn Garden City, UK) or recombinant FSH (Gonal-F; Serono or Puregon; Organon, Cambridge, UK) depending on age and previous response to ovarian stimulation. Human chorionic gonadotrophin (HCG) 10,000 IU (Profasi; Serono or Pregnyl; Organon) was administered to induce oocyte maturation when at least three follicles had reached a mean diameter of 18 mm or more.

Transvaginal follicular aspiration was carried out 34–36 h after HCG injection using an ultrasound scanner with a 6.5 MHz probe (Hitachi EUB 525, Tokyo, Japan). Cumulus–oocyte complexes were isolated from follicular aspirates, washed and incubated at 37°C in an atmosphere of 5% CO_2 in air.

Embryo culture and selection

Embryos were cultured in individual droplets (100 µl) of Sydney IVF Fertilization and Cleavage medium under oil (Cook IVF, Australia). Normally fertilized zygotes, with two clearly delineated pronuclei at 16–20 h post-insemination/microinjection were observed again at 25–27 h post-insemination/microinjection and categorized according to their developmental stage: 1 cell (pronuclei still visible), 1 cell (pronuclei not visible) or 2 cell (see **Figure 1**). Embryos that did not fit into any of these categories were categorized as 'other', and included those embryos with 1 pronucleus visible, eight embryos that had developed a third pronucleus (these were subsequently discarded), embryos that were in the process of division, and those embryos that had divided beyond the 2-cell stage.

Embryos were scored for number of blastomeres and

degree of fragmentation at 42–44 h (day 2) post-insemination/microinjection and selected for transfer on morphological grounds at this time. Embryo transfer took place 46–50 h post-insemination/microinjection (day 2). Embryos were graded on a scale of 1–4 where grade 4 represents best quality. Where available, grade 4 or 3 embryos were transferred, and if embryos appeared similar on day of transfer, those that had undergone cleavage by 25–27 h were chosen preferentially.

Statistical analysis

Only cycles in which all the embryos transferred were at the same developmental stage at the 25–27 h assessment were used for analysis.

Frequency data were tested using the G-test (log-likelihood ratio test) and William's correction was applied as appropriate (Sokal and Rohlf, 1995).

Results

During the course of the study, a total of 2447 embryos from 352 cycles were assessed for early cleavage at 25–27 h after insemination/microinjection (**Table 1**). Of these, 615 (25.1%) were at the 2-cell stage (group C), 757 (30.9%) had no visible pronuclei (group B), 967 (39.5%) were at the pronuclear stage (group A), and 108 (4.4%) did not fit into any of the above three categories and were therefore not included in the subsequent analysis (**Table 1**). There was no significant difference in the proportion of embryos in the three groups when comparing IVF and ICSI cycles (**Table 1**), although there were more early cleaving embryos in the ICSI group.

Overall, early cleavage was observed in 220/352 (62.5%) of cycles. In 41/352 (11.6%) cycles, all embryos still had visible pronuclei, and in 91/352 (25.9%) of cycles, a proportion of embryos had no visible pronuclei but early cleavage was not evident.

Embryo-specific data

The three morphologically distinct groups of embryos observed at 25–27 h post-insemination/injection, pronuclei present (group A), pronuclei absent (group B) and early cleaving (group C), were scored again at 42–44 h post-insemination/injection and assessed for embryo development. Twenty-nine percent of group A embryos had reached the 4-cell stage whereas over 80% of group C embryos and over 65% of group B embryos had 4 cells at this time (**Table 2** and **Table 3**), suggesting that both early cleavage and absence of pronuclei at 25–27 h post-insemination/injection were related to a higher cell number on day 2.

Cycle-specific data

Embryos were selected for replacement on the basis of cell number and cellular morphology at the time of transfer and presence of early cleavage was used as a further discriminating feature in some cases. When considering developmental capacity of the various groups of embryos, pregnancy and implantation rates were assessed only in cycles where embryos selected for transfer were at an equivalent stage when scored at 25–27 h post-insemination/injection. This occurred in 211 cycles (60% of total) and further analysis was carried out on these cycles (**Table 4**). Embryo transfers were divided into three groups; those where all the embryos transferred had been at the pronuclear stage at the 25–27 h check (A), those where all the embryos had no visible pronuclei at this time (B) and those where all embryos transferred had undergone early cleavage (C). The majority were two embryo transfers (75.8%).

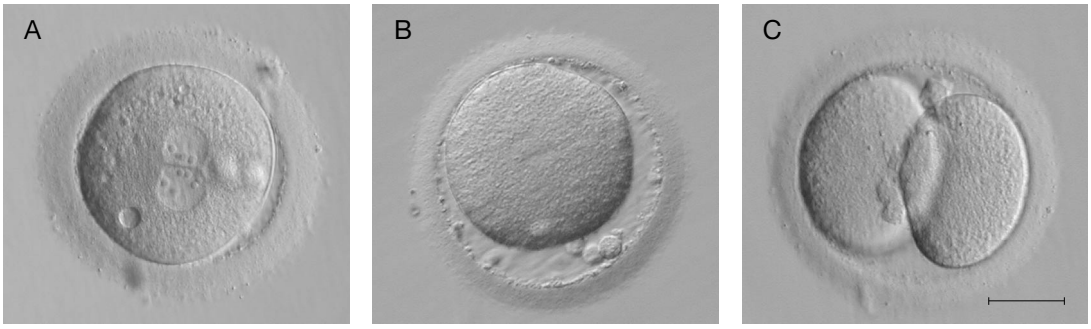

Figure 1. Cross-sectional photos of embryos 25–27 h post-insemination. (**A**) pronuclear, (**B**) syngamy, (**C**) 2-cell. Scale bar = 30 μm.

Table 1. Developmental stage at 25–27 h (values in parentheses are percentages).

	A (pronucleate)	*B (syngamy)*	*C (divided)*	*Other*	*Total*
No. embryos	967 (39.5)	757 (30.9)	615 (25.1)	108 (4.4)	2447
No. IVF	610 (41.6)	455 (31.2)	335 (22.8)	65 (4.4)	1465
No. ICSI	357 (36.4)	302 (30.8)	280 (28.5)	43 (4.4)	982

No significant difference was found in mean age, number of oocytes, basal FSH or mean number of embryos between the groups (**Table 4**). However, a significantly higher percentage of clinical pregnancies was achieved after transfer of early cleaving embryos (group C) than was achieved after transfer of embryos which had intact pronuclei at the time of scoring (group A; $P < 0.001$). The IPR was also significantly higher for group C embryos than for group A embryos ($P < 0.001$) and for group B compared with group A embryos ($P < 0.05$). Pregnancy outcome for the three groups is shown in **Table 5**. There is an increase in biochemical pregnancy rate following transfer of group A embryos, but this increase was not statistically significant.

Discussion

The data presented in this paper confirm previous reports suggesting a positive correlation between early cleavage at 25–27 h post-insemination/injection and increased implantation potential (Shoukir *et al*., 1997; Sakkas *et al*., 1998; Bos-Mikich *et al*., 2001; Lundin *et al*., 2001; Lundqvist *et al*., 2001; Fenwick *et al*., 2002). A clinical pregnancy rate of 43.2% was achieved in cycles where only early cleaving embryos were replaced, compared with 17.2% where all embryos transferred were still at the pronuclear stage at 25–27 h ($P < 0.001$). This equated to an IPR of 32.1% in cycles where two early cleaving embryos were transferred compared with 10.3% for transfers involving only pronucleate embryos. The study reported here compares favourably with other studies seeking to relate the presence of early cleavage to enhanced developmental potential in human embryos. In the first study to demonstrate such a correlation, Shoukir *et al*. (1997) reported a pregnancy rate of 33.3% in IVF cycles in which early cleaving embryos were replaced compared with 14.75% after transfer of embryos which had not demonstrated early cleavage. Subsequent studies reported pregnancy rates of between 25.9% (Sakkas *et al*., 1998) and 55% (Bos-Mikich *et al*., 2001) after replacement of early cleavage embryos and between 3.2% (Sakkas *et al*., 1998) and 25% (Bos-Mikich *et al*., 2001) when embryos with no early cleavage were

Table 2. IVF day 2 embryo development (values in parentheses are percentages).

	A	*B*	*C*	*Other*
Number of embryos	610	455	335	65
2-cell (%)	239 (39.1)	48 (10.5)	10 (2.9)	7
3-cell (%)	90 (14.7)	45 (9.8)	12 (3.5)	7
4-cell (%)	188 (30.8)	298 (65.4)	276 (82.3)	37

Table 3. ICSI day 2 embryo development (values in parentheses are percentages).

	A	*B*	*C*	*Other*
Number of embryos	357	302	280	43
2-cell (%)	158 (44.2)	25 (8.2)	4 (1.4)	8
3-cell (%)	44 (12.3)	32 (10.9)	13 (4.6)	3
4-cell (%)	95 (26.6)	208 (68.8)	236 (84.2)	19

Table 4. Comparison of treatment cycle parameters for patients with embryos transferred all group A (2PN at 26 h), B (syngamy) or C (early cleavage). Values are mean ± SD unless otherwise stated.

Parameter	*Group A*	*Group B*	*Group C*	P-*value*
No. of cycles	58 (27.5)	58 (27.5)	95 (45.0)	–
No. IVF cycles	42 (31.8)	38 (28.8)	52 (39.4)	–
No. ICSI cycles	16 (20.3)	20 (25.3)	43 (54.4)	–
Maternal age (years)	34.5 ± 4.2	33.9 ± 3.9	33.1 ± 4.4	NS
No. oocytes	10.6 ± 5.8	12.2 ± 8.0	12.6 ± 6.8	NS
No. embryos	5.17 ± 3.30	7.26 ± 5.71	7.57 ± 4.27	NS
FSH (U/litre)	7.0 ± 2.1	6.2 ± 1.7	6.3 ± 2.0	NS
Pregnancies per transfer (%)	13/58 (22.4)	21/58 (36.2)	51/95 (53.7)	A vs C <0.001
Clinical pregnancies (%)	10/58 (17.2)	18/58 (31.0)	41/95 (43.2)	A vs C <0.001
Implantation rate (%)	12/117 (10.3)	24/107 (22.4)	60/187 (32.1)	A vs B <0.05, A vs C <0.001

Values in parentheses are percentages.

Table 5. Biochemical pregnancies and miscarriages (values in parentheses are percentages).

	A	*B*	*C*
Biochemicals	3/13 (23)	1/21 (4.8)	7/51 (13.7)
Miscarriage	1/13 (7.7)	1/21 (4.8)	5/51 (9.8)

transferred. Although these studies were not identical in design, there seems to be a consensus in all reports published so far that early cleavage, when present, is related to increased developmental potential in early human embryos.

It has been suggested that to categorize embryos for the presence of early cleavage successfully, the selection of a critical time-point for observation is essential to maximize the differences between embryos and that this time-point may be slightly different in embryos created by ICSI compared with those generated by IVF (Shoukir *et al*., 1997). In this study, the objective was to assess whether clinically relevant data could be obtained in both IVF and ICSI cycles using one static observation, thus minimizing workload and disturbance to embryos during this crucial period. Since early cleavage was observed in only around 25% of embryos (this report and Lundin *et al*., 2001), further discrimination of the remaining embryos was achieved by recording the fate of those that had no discernible pronuclear membrane at the time of scoring which accounted for 30.9% of the total. The extra discrimination afforded by this categorization was especially useful in cycles where there were no early cleaving embryos, but there were embryos with no visible pronuclei (25.9% of cycles during this study). Following pronuclear breakdown, chromosomes condense and align on the metaphase spindle prior to the first cleavage, therefore embryos without pronuclei could reasonably be assumed to be progressing at a faster developmental rate than those with intact pronuclei, but more slowly than those which had already divided. This supposition is borne out by the results reported here, in which a clinical pregnancy rate of 31% and IPR of 22.4% was achieved when embryos that had no visible pronuclei 25–27 h post-insemination/injection were replaced compared with a clinical pregnancy rate of 17.2% and IPR of 10.3% for pronucleate embryos. Although this was not a randomized study, prospective, randomized investigations have found that there is no natural bias in favour of selecting early cleaving embryos for transfer, i.e. the best quality pronucleate embryos were indistinguishable from the best quality early cleavage embryos (Fenwick *et al*., 2002). The study of Fenwick *et al*. also suggested that when looking at blastocyst formation of spare embryos, improved development potential of embryos was embryo-specific rather than patient-specific. Indeed, early cleavage has been shown to be an independent predictor of birth in ICSI cycles (Lundin *et al*., 2001).

It is unlikely that observation of embryos at 25–27 h post-insemination/injection will replace morphological criteria on the day of embryo transfer as a method of embryo selection, but it will provide more information about the potential of the embryo to achieve implantation. In cases where embryos within a cohort appear very similar on the day of transfer, this extra observation is particularly useful. As such, scoring for the presence of cleavage and pronuclei could be used in conjunction with other established ways of grading embryos to try to reduce the number of embryos transferred in any one cycle in order to reduce the risk of multiple pregnancy.

It has been suggested that early cleavage may simply be the result of precocious fertilization by a few oocytes that were more mature at the time of insemination and therefore primed to fertilize earlier (Shoukir *et al*., 1997). However, early cleavage was also seen in a subset of embryos after ICSI, when timing of fertilization could be precisely evaluated. This led both Shoukir *et al*. (1997) and Sakkas *et al*. (1998) to conclude that early cleavage may be the result of unknown factor(s) within the embryo which favours or promotes early division and may subsequently also improve embryo viability. It is interesting to note that in the present study, early cleavage was independent of both female age and FSH concentrations, leading us to conclude, like Fenwick *et al*. and Lundin *et al*. (see above), that early cleavage may indeed be an independent intrinsic property of a particular group of embryos. There are several important factors that may influence the rate of cleavage in human embryos. These include expression of developmentally regulated genes (Warner and Brenner, 2001; Warner *et al*., 2002), chromosomal abnormalities (Munné *et al*., 1995) and culture environment (reviewed in Boiso *et al*., 2002) among others. However, one factor which influences directly both the ability to complete the first cleavage division and its timing, is the number and quality of calcium transients generated at fertilization. The release in intracellular calcium is a fundamentally important signalling event in fertilization and development (Kline and Kline, 1992; Stricker, 1999), and recent investigations have indicated that calcium transient number, frequency, amplitude and duration can regulate many individual cellular processes (Ducibella *et al*., 2002). Using the mouse oocyte as a model it has been shown that calcium oscillations in fertilized eggs may regulate not only short term (Ozil, 1990), but also long-term developmental events (Ozil and Huneau, 2001). By precisely manipulating the number of transients experienced by individual mouse eggs using pulsatile electrical stimulation, it has also been demonstrated that each of the many events required for successful fertilization, such as extrusion of cortical granules, resumption of the cell cycle and recruitment of maternal mRNAs, is differentially sensitive to the number of calcium transients experienced, for both its initiation and completion (Ducibella *et al*., 2002). It has been proposed that the sperm introduces a specific protein factor into the egg cytoplasm after gamete membrane fusion that is directly responsible for initiating the Ca^{2+} oscillations that lead to fertilization of the egg (Swann *et al*., 2001). This protein has been isolated and injected into mouse eggs resulting in Ca^{2+} oscillations (Saunders *et al*., 2002). Since the amount injected determines the frequency of Ca^{2+} (Cox *et al*., 2002; Saunders *et al*., 2002), it could be proposed that the development of each individual embryo could be profoundly influenced by the capacity of an individual spermatozoon to induce an optimal pattern of calcium transients at fertilization. This hypothesis has received support from studies in which alteration of Ca^{2+} oscillation number, amplitude or frequency in parthenotes can profoundly affect the ability of mouse zygotes to form pronuclei (Vitullo and Ozil, 1992) and the relative numbers of cells in the inner cell mass and trophectoderm (Bos-Mikich *et al*., 1997). In addition, the ability of the oocyte to respond to the Ca^{2+} stimulus is also likely to vary markedly as the oocyte itself

acquires the ability to support repetitive Ca^{2+} transients during maturation (Carroll *et al*., 1994), suggesting that oocyte maturity may also be a major factor influencing the timing of first cleavage.

In this study, it has been confirmed that the assessment of early cleavage to the 2-cell stage can be used as an indication of embryo quality and that embryos that cleave early have an increased chance of implanting and giving rise to a pregnancy. Scoring for the presence of early cleavage is quick and objective and provides extra information for the embryologist when faced with several equivalent embryos at the time of transfer. Adding the extra category of no visible pronuclei provides the chance for additional discrimination without any further manipulation, in what appears a critical time window for the developing embryo. This additional categorization may be particularly important when no early cleaving embryos are present in the cohort or in IVF cycles where the precise timing of fertilization is more difficult to ascertain. Since Lundin *et al*. found early cleavage to be an independent predictor of both pregnancy and birth in ICSI cycles, but not in IVF cycles (Lundin *et al*., 2001), additional categorization of embryos may help overcome this problem.

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