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Cleavage anomalies in early human embryos and survival after prolonged culture in-vitro

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This study examines the relationship between common morphological anomalies of cleaving embryos and their ability to form apparently normal blastocysts *in vitro*. The impact of cleavage rate, fragmentation, and multinucleation on compaction, cavitation, along with inner cell mass and trophectoderm formation has been assessed. The study population consisted of 102 patients who elected or were selected to have a day 5 embryo transfer. Clinical pregnancy and implantation rates were 66.7 and 49% respectively. Slow and fast cleavage had a significant negative association with normal blastocyst formation. Only 13.8% (67/484) of embryos with <7 cells and 27.5% (25/91) of those with >9 cells on day 3 formed blastocysts with apparently normal morphology, compared to 41.9% (252/602) with 7–9 cells on day 3 ($P < 0.001$). Fragmentation had a negative impact on normal blastocyst formation. Embryos with >15% fragmentation formed normal blastocysts at a significantly lower rate (46/279; 16.5%) than embryos with 0–15% fragmentation (311/935; 33.3%) ($P < 0.001$). Furthermore, the pattern of fragmentation was associated with blastocyst formation. Type IV fragmentation led to a significant reduction in blastocyst formation (25/170 or 14.7%), compared to types I, II and III which performed much better (38.6, 32.9 and 32.4% respectively). Only 15.9% (22/138) of embryos with one or more multinucleate cells on day 2 and/or 3 formed normal blastocysts compared with 31.9% (335/1051) ($P < 0.001$) of those without multinucleation. Collectively, the data suggest that cleavage anomalies, some of which do not preclude development after short-term culture, may reduce the developmental competence of embryos after prolonged culture.

Key words: blastocyst/cleavage rate/fragmentation degree/fragmentation pattern/multinucleation

Introduction

While 70% of eggs fertilized *in vitro* undergo the first three cleavage divisions during 3 days in culture, fewer than half advance to cavitation after 5 days (Gardner *et al*., 1998a,b; Jones *et al*., 1998; Behr *et al*., 1999), and about one-third form morphologically optimal blastocysts with a well-defined inner cell mass (ICM), a cohesive trophectoderm (TE), and full expansion (Racowsky *et al*., 1999).

It has been proposed that prolonged culture allows development of 'normal' embryos with implantation potential, while 'abnormal' and non-viable embryos arrest before or shortly after the onset of genomic activation (Huisman *et al*., 1994; Dawson *et al*., 1995; Janny and Ménézo, 1996; Gardner and Lane, 1997).

Indeed, a number of intrinsic factors have been shown to influence survival to the blastocyst stage in extended culture *in vitro*; among them are sperm quality (Janny and Ménézo, 1994; Jones *et al*., 1998), the aetiology of infertility (Ménézo *et al*., 1995), and maternal age (Janny and Ménézo, 1996; Schoolcraft *et al*., 1999).

Cleavage patterns have also been linked to blastocyst formation (Bolton *et al*., 1989; Wiemer *et al*., 1995; Balakier and Cadesky, 1997; Rijnders and Jansen, 1998). This, together with the link between chromosomal abnormalities and aberrant early embryo morphology (Pellestor *et al*., 1994; Munné *et al*., 1994, 1995; Sadowy *et al*., 1998), as well as the observed viability of in-vitro grown blastocysts (Gardner *et al*., 1998a,b, 2000; Schoolcraft *et al*., 1999) lend support to the 'survival of the fittest' proposal.

However, the relationship between morphology, chromosomal integrity, embryogenesis *in vitro*, and viability is clearly more complex. Embryos with normal morphology may be chromosomally abnormal, but can reach the blastocyst stage; on the other hand, many chromosomally normal embryos with atypical or normal morphology fail to undergo differentiation in extended culture (Sandalinas *et al*., 2000). There is also some clinical evidence that suggests a loss of development potential with extended culture of compromised embryos. It has been reported that patients with no 8-cell embryos on day 3 had a 33% pregnancy rate after day 3 transfers, but failed to achieve pregnancies after day 5 transfers (Racowsky *et al*. 1999).

So, extrinsic factors such as prolonged culture itself may also contribute to the loss of in-vitro-generated embryos over time in culture. But, it is so far unclear which embryos may be affected and to what extent. In the present study, the impact of cleavage rate, fragmentation, and multinucleation on compaction, cavitation, along with ICM and TE formation after prolonged culture is assessed. Morphological abnormalities in the pattern of blastulation are described. The relationship between morphological profiles of blastocysts and implantation after day 5 transfer was also evaluated. The findings have

important implications for the application of day 5 transfer after IVF.

Materials and methods

Patients and follicular stimulation

Patients were treated between October 1997 and March 1999 at the Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center. The Internal Review Board of Saint Barnabas Medical Center approved the review of these patient records. The study population consisted of 102 patients (including 38 oocyte recipients) who elected or were selected to have embryos transferred on day 5 of development, mainly to avoid high order multiple pregnancy. The mean (± SD) ages of regular patients and oocyte recipients were 33.89 ± 3.7 and 40.06 ± 4.21 years respectively.

Procedures during which embryos were pooled in groups ($n = 11$) were excluded from the study, as the embryos could not be individually tracked during culture.

A standard down-regulation protocol included 1 mg of Lupron (TAP Pharmaceuticals, Lake Forest, IL, USA) starting on day 21 of the cycle prior to the stimulation cycle. On day 3 of menses, suppression was verified by both ultrasound and blood oestradiol and progesterone concentrations. On the day of gonadotrophin start (recombinant FSH; Gonal-F; Serono Laboratories, Norwell, MA, USA), Lupron dose was decreased to 0.25 mg and continued until the day of human chorionic gonadotrophin (HCG) administration. The usual daily dose of gonadotrophin was 4 ampoules, given singly on the evening of the first day, and split between morning and evening thereafter. Follicular development was monitored by ultrasound and blood hormone concentrations. When the lead follicle reached 16–17 mm in diameter and a minimum of four mature follicles was present, HCG was administered. Oocyte retrieval was scheduled to 36 h after HCG.

Gamete and embryo culture

Oocytes were collected in HEPES-buffered human tubal fluid medium (HTF) (Quinn *et al*., 1985). After cumulus dissection and wash, they were placed in 100–200 µl droplets of protein-supplemented HTF under mineral oil (Squibb, Princeton, NJ, USA). Protein supplementation was provided either by 10% heat-inactivated maternal serum, 6% plasmanate (Miles Inc., Elkhart, IN, USA) or 6% synthetic serum substitute (SSS; Irvine Scientific, Irvine, CA, USA).

Normally fertilized oocytes with two pronuclei were cultured individually in 100 µl droplets of HTF supplemented with protein, or in droplets of G1.2 (courtesy of D.Gardner; nine patients only). They remained in these media until early afternoon of day 3, when they were placed in 100 µl drops of G2.2 (courtesy of D.Gardner or purchased from Scandinavian IVF Science, Gothenburg, Sweden) or S2 (Scandinavian IVF Science) following a 5-drop rinse in the appropriate medium. A second changeover to fresh G2.2 or S2 was done on the morning of day 5, after embryo evaluation and before embryo transfer. Embryo transfers were scheduled between noon and 14.00 h on day 5.

A total of 1395 zygotes from the 102 patients was cultured, of which 112 were cryopreserved on or before day 3. An additional 69 embryos were discarded before reaching day 5, as they were judged to have completely arrested. Thus the total number of embryos cultured to day 5 was 1214. The number of embryos in the cleavage rate analyses differed slightly from this number, since the pertinent information was not available in the database for all the embryos in the study.

Table I. Classification of embryonic development on days 4 and 5

Developmental event	Day 4	Day 5
Compaction		
Complete	N	S
Incomplete	N	S
Regional	S	S
Fusion-like	S	S
None	S	A
Cavitation		
Single cavity	[a]	N
Beginning	N	S
Large vacuoles	A	A
Multiple cavities	A	A
Inner cell mass formation		
Distinct/organized	[a]	N
Forming	[a]	S
Large cells	[a]	S
Small mass	[a]	S
None	[a]	A
Trophectoderm organization		
Cohesive	[a]	N
Large cells	[a]	S
Irregular	[a]	A

[a]Not expected on this day.
N = normal; S = suboptimal; A = abnormal.

Embryo evaluation

Fertilization was confirmed ~14 hours post insemination. On days 2, 3, 4 and 5 of development, embryos were evaluated on an Olympus IX70 inverted microscope (Olympus America, Melville, New York, NY, USA), equipped with Hoffman Modulation Optics (Narishige, Tokyo, Japan). Total magnification was ×600. Cell number, degree and pattern of fragmentation, and the presence of multinucleate blastomeres were recorded on days 2 and 3 of development.

Cell numbers of embryos on days 2 and 3 were adjusted according to individual time in culture after oocyte retrieval using the following formula:

(cell number/hours in culture)×average hours in culture for all embryos in the database.

The average number of hours in culture from oocyte retrieval to day 2 and day 3 was 48.28 and 71.68 respectively.

The degree of fragmentation was expressed as a percentage and defined as the embryonic volume occupied by anucleate cytoplasmic fragments (Puissant *et al*., 1987). Fragmentation pattern was defined based on spatial distribution and relative size of the fragments, as described previously (Alikani and Cohen, 1995; Warner *et al*., 1998; Alikani *et al*., 1999).

Development on days 4 and 5 was recorded in detail during the study but was not classified until data analysis. At that time, embryos were classified according to the criteria listed in Table I. As is the nature of all morphological classification systems, the one proposed here is subjective, but based on our own observations as well as those of others on morula and blastocyst morphology in the human (e.g. Cohen *et al*., 1985; Hardy *et al*., 1989; Dokras *et al*., 1991, 1993; Van Blerkom, 1993; Gardner and Schoolcraft, 1999; Ménézo *et al*., 1999). Our analyses do not include day 6 development since (i) the majority of the blastocysts in this study, as in other studies (Gardner *et al*., 1998a,b) formed on day 5, (ii) embryos that did not undergo differentiation by day 5 rarely formed normal blastocysts on day 6. This is not to say that day 6 blastocysts do not have an implantation potential, rather that in the context of this study, timing was an important factor in definition of normal development.

Compaction was expected to occur on day 4 (Nikas *et al*., 1996);

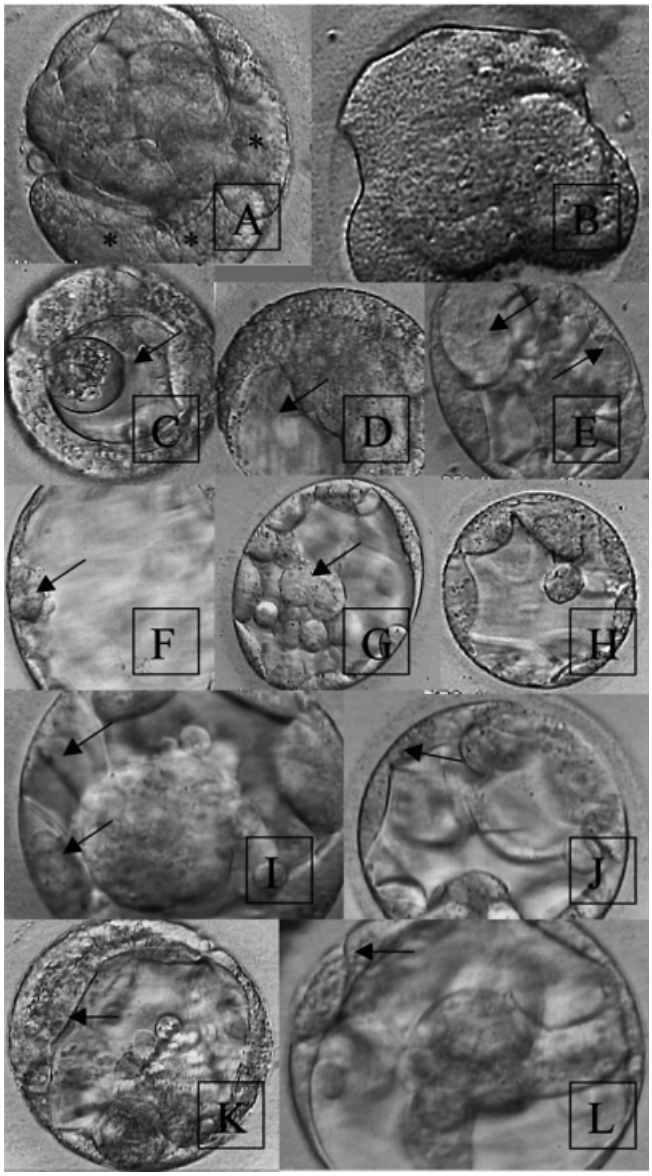

Figure 1. Various forms of compaction, cavitation, inner cell mass formation, and trophectoderm organization (**A**–**L**). Regional compaction occurred when several blastomeres (marked with asterisks) were excluded from the morula (**A**); complete disappearance of inter-blastomeric spaces was termed 'fusion-like compaction' and was considered abnormal (**B**); large vacuoles, sometimes with internalized excluded cells or fragments (**C**, arrow), small cavities (**D**, arrow), and multiple cavities (**E**, arrows) were all considered abnormal forms of cavitation; a small mass of inner cells (**F**, arrow) or few large and disorganized inner cells (**G**, arrow) were suboptimal formations, while the absence of an inner cell mass (ICM) (**H**) was abnormal. Large trophectoderm (TE) cells, few in number (**I** and **J**, arrows), were considered suboptimal. Irregular forms of ICM and TE cells (**K** and **L**) were considered abnormal.

a delay of 1 day in compaction was considered an indication of slow development and thus suboptimal. Absence of compaction on day 5 was considered abnormal. Compaction on both days 4 and 5 was defined as complete, incomplete, regional, or fusion-like. Complete or incomplete compaction involved all cells within the embryo, with all cells appearing flattened and cell boundaries becoming vague (complete) or only some cells demonstrating this morphology and cell borders still recognizable (incomplete). Regional compaction (Figure 1A) marked exclusion of one or more cells from the compacted morula. A less common form of compaction was termed 'fusion-like' and involved the complete disappearance of intercellular spaces not by flattening of cells, but by what appeared as 'fusion' of cells (Figure 1B). The extent to which these morphologies determined the potential of the morula for development needs further assessment; but for the purposes of this study and based on the likelihood of these embryos to form normal blastocysts on day 5, we classified regional, fusion-like, and delayed compaction (one full day's delay) as suboptimal.

It was not unusual to see the beginning of cavitation on day 4. However, a single defined cavity was never observed before day 5. If a cavity was beginning to form on day 5, this was suboptimal, but included as normal. The persistence of large vacuoles, small and multiple cavities on day 5 was considered abnormal (Hardy *et al*., 1989; Dokras *et al*., 1991, 1993) (Figure 1C, D and E respectively).

A distinct ICM, organized as a compacted mass of numerous cells, was considered normal. If the inner cells formed a small mass, or if the mass comprised loosely packed cells, the formation was suboptimal but considered normal (Gardner and Schoolcraft, 1999) (Figure 1F and G respectively). The absence of an ICM was abnormal (Hardy *et al*., 1989) (Figure 1H). A cohesive layer of numerous tightly packed cells in the TE was normal; large TE cells were suboptimal but again considered normal (Figure 1I and J). Highly irregularly arranged ICM and/or TE cells were considered abnormal (Figure 1K and L). A blastocyst was considered morphologically normal if it contained either a normal or suboptimal ICM in combination with either a normal or suboptimal TE on day 5 (see Table I). The appearance of either an abnormal ICM or an abnormal TE, or a full day's delay in differentiation placed the day 5 embryo in the abnormal category. Ten representative blastocysts of normal appearance are shown in Figure 2; six of these have become babies.

Data collection and analysis

Data for this study were collected and analysed using the EggCyte database (EggCyte™; ART Institute of NY and NJ, Livingston, NJ, USA, 1995–2000). Our group maintains a computer network with both paper and on-line database record-keeping with terminals in the laboratory and elsewhere. Data are recorded at the point of generation and afterwards confirmed with the paper records. Using MS-Access (Microsoft Corp., Seattle, WA, USA), we have created a suite of tables, forms, program functions and reports tailored to the requirements of clinical embryology for individual oocyte and embryo tracking. Like any relational database, the tables link the relevant records by indexed numeric fields common to each.

After retrieval, oocytes are numbered and tracked individually with separate pages or tables for each day of development, just as in a daily journal. When embryos are replaced, separate records for each are again created and linked to the record of the replacement procedure. As pregnancy results become available, they are entered in a further table, always linked by procedure number. Data for the analyses in this study were extracted by creating selection lists or queries from relevant tables in the database. EggCyte has over 40 data tables but this study has used about 14 altogether. Over 300 queries of varying complexity with Structured Query Language (SQL) and Visual Basic programming functions (Microsoft Corp.) were used to generate the data presented here.

Differences in the number of embryos compacting, cavitating, and forming blastocysts in each fragmentation group as well as in each cleavage group were tested for significance using χ^2

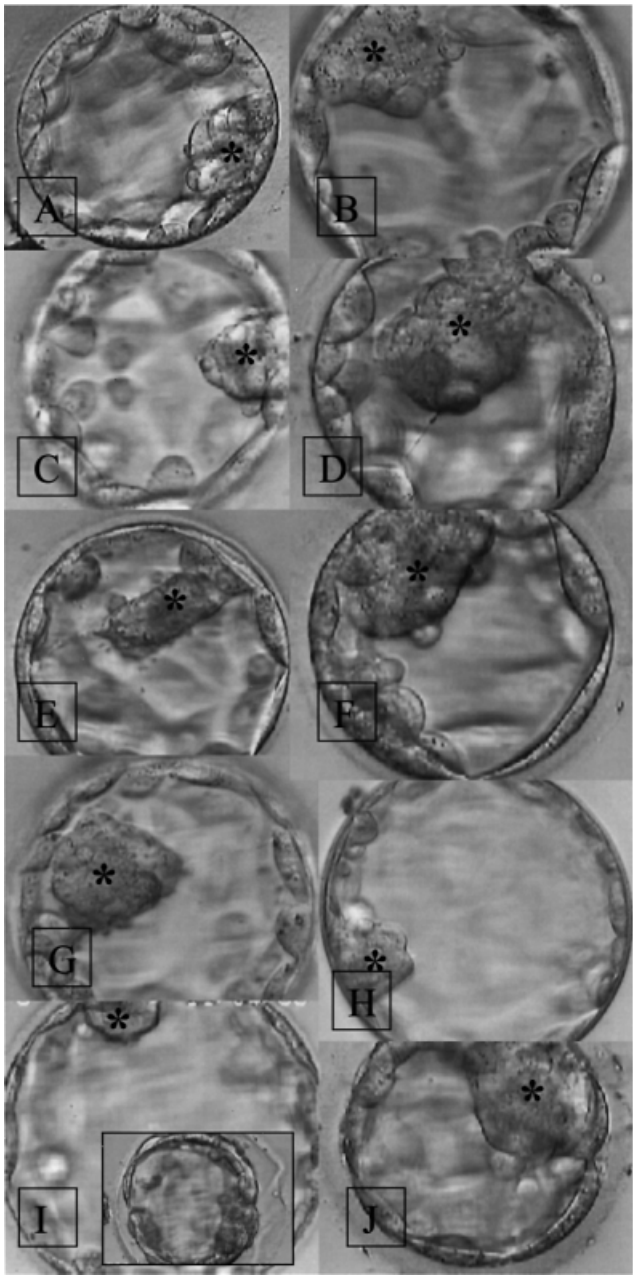

Figure 2. Representative day 5 embryos with normal morphology. The inner cell mass (ICM) has been marked in each panel with an asterisk (*). A remarkable variation in size and shape of the ICM, as well as of trophectoderm cells, is visible. Embryo in **A** (delivered male) was an 8-cell without fragmentation on day 3. Embryo in **B** was an 8-cell with 15% fragmentation type III and did not establish a heart beat. Embryo in **C** (delivered male) was an 8-cell with 5% fragmentation type I. Embryo in **D** (delivered male) was an 8-cell with 10% fragmentation type III. Embryos in **E** and **F** were 9-cell with 20% fragmentation type III and 8-cell with 15% fragmentation type III; they both implanted, but only a single female was delivered. Embryo in **G** was a 14-cell without fragmentation and did not implant. Embryo in **H** was an 8-cell with 15% type III fragmentation and did not implant. Embryo in **I** (delivered female) was an 11-cell without fragmentation; the inset shows the same blastocyst after collapse of the blastocoelic cavity; the zona pellucida is thin and open at the 3 o'clock position. Embryo in **J** (delivered female) was an 8-cell without fragmentation.

Table II. Overall clinical outcome for day 5 transfers

	n
Total no. of procedures	102
No. of procedures with FHB >0	68
Clinical pregnancy rate (%)	66.7
No. delivered[a]	41
No. ongoing[a]	16
No. of eggs retrieved (average)	2146 (21.0)
No. of 2 PN zygotes	1395
No. of blastocysts	576 (44.9%)[b]
No. of embryos replaced	208
No. of FHB	102
Implantation rate (%)	49

[a]As of March 1, 2000; 11 miscarriages.
[b]Blastocyst formation rate reflects 114 embryos that were cryopreserved before or on day 3.
FHB = fetal heart beat; PN = pronuclear.

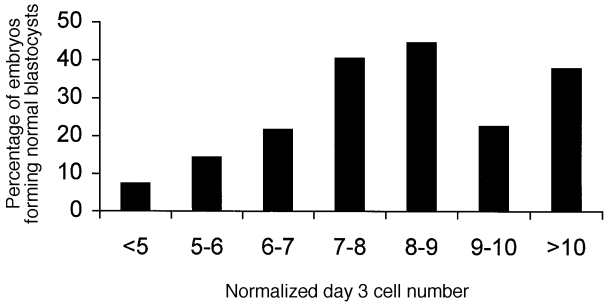

Figure 3. Normalized cell numbers on day 3 of development and the incidence of normal blastocyst formation on day 5. Embryos with <7 cells or >9 cells formed normal appearing blastocysts significantly less frequently than those with 7–9 cells ($P < 0.001$).

analysis. Differences between mean cell numbers were analysed using analysis of variance. $P < 0.05$ was considered significant.

Results

Overall clinical results

Table II is a summary of clinical outcome for the 102 cases reported here. Clinical pregnancy rate with confirmed fetal heart activity on ultrasound was 66.7% (68/102). Implantation rate i.e. percentage of transferred embryos represented by fetal heart beats (FHB) was 49.0% (102/208). The average number of embryos transferred per patient was 2.06. Five patients received one embryo, 88 received two embryos, and nine patients received three embryos. Total blastulation rate was 44.9%; and normal blastocyst formation rate was 30.6% (371/1214) (some were suboptimal; see Table I, Figure 2, and materials and methods section). The majority of the blastocysts were obtained on day 5. All transfers were performed on day 5.

Development rate and blastocyst formation

Figure 3 is a summary of the data for a total of 1177 embryos in the cleavage rate analysis. The rate of development was an important determinant of normal blastulation. Only 7.4% (15/204) of the embryos with <5 cells and 14.3% (17/119) of those with 5-6 cells on day 3 formed a morphologically normal

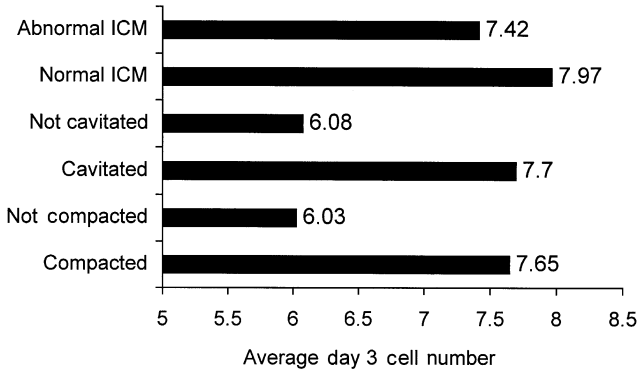

Figure 4. Average cell number of day 3 embryos and blastulation. Embryos that successfully underwent normal compaction, cavitation, and inner cell mass (ICM) formation had significantly higher cell numbers than those embryos that failed to complete these developmental stages ($P < 0.001$).

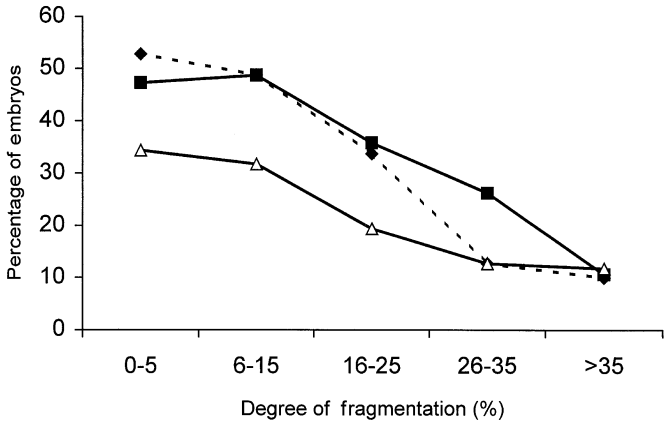

Figure 5. The relationship between fragmentation degree and normal compaction (■), cavitation (▲), and blastocyst formation (△). When fragmentation exceeds 15%, the rates of normal compaction, cavitation and blastocyst formation are significantly reduced ($P < 0.001$).

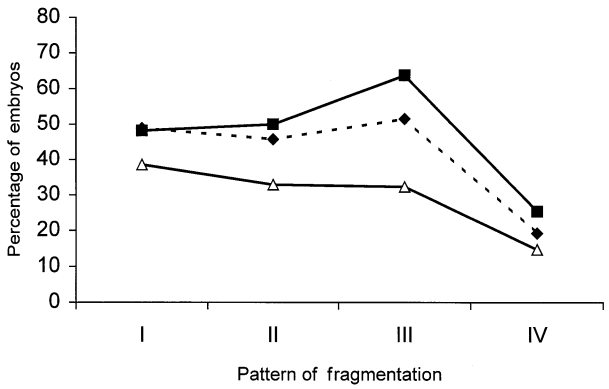

Figure 6. The relationship between pattern of fragmentation and normal compaction (■), cavitation (▲), and blastocyst formation (△). Embryos with type IV fragmentation have significantly lower rates of compaction, cavitation and blastocyst formation than those with fragmentation types I, II and III ($P < 0.001$).

blastocyst on day 5. The proportion of embryos developing into normal-appearing blastocysts increased significantly among embryos with 6–7 cells (35/161; 21.7%), 7–8 cells (157/389; 40.4%), 8–9 cells (95/213; 44.6%), 9–10 cells (14/62; 22.6%), and >10 cells (11/29; 37.9%) ($P < 0.001$).

When divided into three groups, embryos with <7 cells, 7–9 cells and >9 cells on day 3, the proportions of normal-appearing blastocysts were 13.8% (67/484), 41.9% (252/602) and 27.5% (25/91) respectively. The proportion of normal-appearing blastocysts was significantly higher among embryos with 7–9 cells compared to embryos with <7 cells ($P < 0.001$) and >9 cells ($P < 0.01$). The average (± SD) cell numbers on day 2 and day 3 for normally compacted day 4 embryos were 3.92 ± 0.75 and 7.65 ± 1.48 respectively. The day 2 and day 3 averages for embryos that formed a cavity on day 5 were 3.95 ± 0.71 and 7.70 ± 1.45 respectively. If a normal ICM had formed, average cell numbers for days 2 and 3 were 3.96 ± 0.49 and 7.97 ± 1.3, respectively. By contrast, cell numbers on both day 2 and day 3 were significantly lower in non-compacted embryos (3.52 ± 1.21 and 6.03 ± 2.15 respectively) and embryos that did not cavitate (3.56 ± 1.30 and 6.08 ± 2.24 respectively) ($P < 0.001$). Embryos that did not form a normal ICM had significantly fewer cells on day 3 (7.42 ± 1.50) ($P < 0.001$). Day 2 cell numbers did not differ between embryos forming a normal ICM compared with an abnormal one (3.96 ± 0.49 versus 3.91 ± 0.78 respectively). The data for day 3 cell numbers are presented in Figure 4.

Fragmentation and blastocyst formation

Figure 5 shows the relationship between the degree of fragmentation and the incidence of normal compaction, cavitation, and blastocyst formation in 1214 embryos available for this analysis. Normal blastocyst formation rate decreased significantly with increasing fragmentation: 33.3% (311/935) among embryos with 0–15% fragmentation was significantly higher than 16.5% (46/279) among embryos with more than 15% fragmentation ($P < 0.001$). The difference was apparent at compaction (51.1 versus 24.6% respectively) and cavitation (47.9 versus 29.3% respectively). When fragmentation exceeded 35%, all processes were severely compromised, and normal compaction (5/50; 10%), cavitation (5/47; 10.6%) and blastocyst formation (6/51; 11.8%) were lowest.

The pattern of fragmentation was also associated with blastulation; this is presented in Figure 6. Blastocyst formation rates were not different among embryos with fragmentation type I (54/140, 38.6%), type II (27/82, 32.9%), or type III (143/442, 32.4%). However, type IV fragmentation led to a significant reduction in normal blastocyst formation (25/170, 14.7%) ($P < 0.001$). The same trend was apparent at compaction and cavitation.

Multinucleation and blastocyst formation

The data for 1214 embryos in this analysis are presented in Table III. The presence of one or more blastomeres with two or more nuclei either on day 2 or day 3 of development had a strong negative association with the ability of the embryo to compact, cavitate, and form a blastocyst. Among the embryos with multinucleation on day 2 and/or day 3, only 30.6% (44/144) compacted normally, 27.5% (38/138) cavitated normally, and 15.9% (22/138) formed normal blastocysts.

Table III. The effect of multinucleation on day 2 and/or day 3 on development beyond day 3

Day 2/day 3 embryo morphology	Normal compaction rate (%)	Percentage not compacted	Normal cavitation rate (%)	Blastocyst formation rate (%)	Average day 2 cell number[a]	Average day 3 cell number[a]
Without MNB	47.0	38.4	45.9	31.9	3.96 (0.7)	7.76 (1.4)
With MNB	30.6	50.7	27.5	15.9	3.53 (1.0)	6.65 (1.96)
P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a](± SD) For normally compacted embryos.
MNB = multinucleate blastomeres.

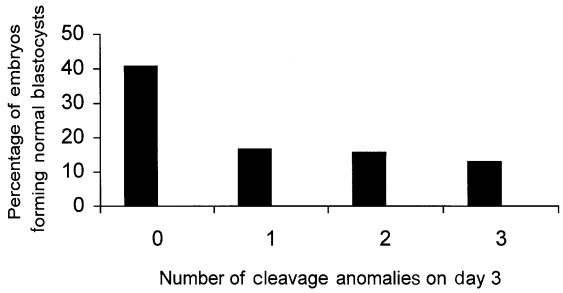

Figure 7. Blastocyst formation rate among embryos with 0, 1, 2 or ⩾3 cleavage abnormalities on day 3 of culture. The highest rate of blastocyst formation was among embryos without abnormalities. The appearance of a single anomaly led to a significant reduction in normal blastocyst formation ($P < 0.001$); this was further reduced with multiple anomalies but not significantly.

The rates of normal compaction, cavitation, and blastocyst formation for embryos without multinucleation were 47% (498/1060), 45.9% (482/1051) and 31.9% (335/1051) respectively. The differences between the two groups were statistically significant ($P < 0.001$).

Multiple cleavage anomalies and blastocyst formation

Blastocyst formation among embryos with multiple anomalies was evaluated. Forty-one per cent of all embryos (493/1214) had one or more of the anomalies described here, that is, on day 3 of development, they had <5 cells, >15% fragmentation, type IV fragmentation, and/or multinucleation. Figure 7 shows blastocyst formation among embryos with 0, 1, 2 or ⩾3 abnormalities. Embryos with no abnormalities formed blastocysts in a significantly higher proportion (294/721 or 40.8%) than those with one anomaly (45/270 or 16.7%), two anomalies (24/153 or 15.7%), or three or more anomalies (8/70 or 11.4%) ($P < 0.001$). Blastocyst formation did not change significantly with increasing number of abnormalities.

Implantation and cleavage profile/morphology of transferred blastocysts

The morphology of the transferred blastocysts was analysed in four patient groups with different pregnancy outcomes (Table IV): (i) 23 patients with no implantation, (ii) 11 patients with a biochemical or an anembryonic pregnancy, (iii) 36 patients in whom at least one of the transferred embryos gave rise to a fetal heart (partial implantation), and (iv) 32 patients in whom all the transferred embryos resulted in fetal hearts. The proportion of non-blastocysts and blastocysts with abnormal morphology in groups (iii) and (iv) with one or more fetal hearts was 15.8% (22/139). This was significantly lower than the proportion in groups (i) and (ii) with complete implantation failure (negative pregnancy result) or with implantation but development failure (biochemical or anembryonic pregnancy) (22/71; 31%) ($P < 0.01$). Moreover, blastocyst abnormalities in groups (iii) and (iv) were limited to the trophectoderm and some slow embryos, but abnormalities in the failed implantation/development groups (i) and (ii) included the ICM and many slow embryos (non-blastocysts).

Of the 208 blastocysts that were transferred, 182 (87.5%) were embryos with ⩽15% fragmentation on day 3, and 26 (12.5%) had >15% fragmentation. Six embryos (2.9%) had multinucleate blastomeres either on day 2 or day 3. In patients of group (iv), all transferred blastocysts implanted (64/62; two pairs of monozygotic twins). Of these blastocysts, only 8/62 (12.9%) had >15% fragmentation and 3/62 (4.8%) exhibited fragmentation pattern IV on day 3. In patients of group (iii), with implantation of 38/75 replaced embryos, day 3 fragmentation in excess of 15% was only found in 8/75 (10.7%), and type IV fragments in 5/75 (6.7%). In groups (i) and (ii) together, where none of the 71 transferred embryos resulted in a fetal heart, the proportion of embryos with >15% fragmentation or type IV fragmentation was similarly low and not different from that found in groups (iii) and (iv). This reflected the low percentage of such embryos reaching the blastocyst stage and the poor morphology of the ones that did since they were rarely selected for transfer.

Implantation potential of embryos with cleavage anomalies after short-term culture

The EggCyte database was searched for homogeneous day 3 transfers involving embryos with either no abnormality or one or more of the anomalies described here. Homogeneous transfers were those where all the embryos transferred belonged in the same morphological category. The resulting implantation rates were compared to blastocyst formation rate in extended culture and the theoretical implantation rate after day 5 transfer (Table V). A similar concept was previously suggested by Edwards and Beard (1999).

Embryos with none of the abnormalities discussed here had an implantation rate of 40.1% (332/828) on day 3. Embryos with cleavage rate anomaly (<5 cells) had an implantation rate of 22.0% (78/354) after day 3 transfer. It must be noted that 1-, 2- or 3-cell embryos were never transferred on day 3, but they were included in the blastocyst formation data. The

Table IV. The incidence of morphological anomalies among transferred blastocysts in four groups of patients with different implantation outcome

Implantation outcome (group)	No. of patients	No. blastocysts transferred	No. FHB	No. abnormal	No. not blastocysts	% abnormal
Failed (i)	23	49	0	4	10	28.6
Anembryonic (ii)	11	22	0	8	0	36.4
Total	34	71	0	12	10	31.0[a]
Some implanted (iii)	36	75	38	7	5	16
All implanted (iv)	32	62	64	7	3	15.6
Total	68	137	102	14	8	15.8[a]

[a]$P < 0.01$ [proportion of abnormal embryos in failed implantation groups (i) and (ii)] compared with the groups with fetal heart activity [(iii) and (iv)].
FHB = fetal heart beats.

Table V. Implantation rate of embryos with specific anomalies after homogeneous day 3 transfer compared with theoretical implantation rate for similar embryos after 5 days in culture

Day 3 cleavage anomaly	Implantation rate (%) (day 3 transfer)	Day 5 blastocyst formation rate (%)	Theoretical implantation rate (%) (day 5 transfer)
<5 cells	22.0	7.4	3.7
>15% fragmentation	17.7	16.5	8.2
Type IV fragmentation	16.5	16.4	7.8
Multinucleate blastomeres	13	14.1	7.0

implantation rate of embryos with >15% fragmentation was 17.7% (40/226). Exclusive transfer of embryos with type IV fragmentation on day 3 resulted in an implantation rate of 16.5% (22/133). Embryos with MNB on days 2 and/or 3 resulted in a low implantation rate of 13.0% (14/108).

The theoretical implantation rate of embryos in prolonged culture was calculated based on the following formula:

(number of normal-appearing blastocysts/total number of embryos cultured)×100×overall implantation rate of blastocysts.

For embryos with slow development and/or fragmentation, the theoretical implantation rate after day 5 transfer appeared to be lower than their actual implantation rate after day 3 transfer, since many were lost during extended culture. Embryos with multinucleation, on the other hand, appeared to perform similarly after short- or long-term culture (Table V).

Discussion

This study demonstrates the relationship between early embryo morphology and blastocyst formation *in vitro*. Slow development (<5 cells on day 3), fragmentation (>15% and/or type IV), and multinucleation (on day 2 and/or 3) all interfered with the formation of apparently normal blastocysts.

The culture system used during this study included culture in HTF on days 0 to 3, then in G2.2 or S2 on days 3.5 to 6. This is a variation on the currently advocated sequential systems, hence the possibility that the observed effects were partly a result of deviation from the established sequence. Although this possibility cannot be excluded, the culture system reported here produced an overall blastocyst formation rate similar to that reported by others (e.g. Gardner *et al.*, 1998a,b). Moreover, a 49% implantation rate reflects the ability of this system to support development of viable blastocysts (Table II). Nevertheless, we are currently evaluating the use of G1.2/G2.2 (commercial preparation) in the blastocyst transfer programme.

The reduced ability to form blastocysts of normal appearance was in many instances already obvious at compaction, which occurs on day 4 of development in the human (Nikas *et al.*, 1996). Regional compaction with exclusion of a number of cells and fragments from the morula occurred among fragmented embryos (Figure 1). Blastocyst formation rate among normally compacted day 4 embryos was 46.8%, but this rate was reduced to 28.6% among regionally compacted embryos and 10.3% among embryos that did not show compaction on day 4 (data not shown). These data suggest that (i) the absence of compaction on day 4 is highly prognostic for normal blastocyst formation, and (ii) while regional compaction by itself does not preclude blastocyst formation, the extent to which exclusion occurs may determine development potential; we did not note the number of excluded cells when regional compaction occurred. Exclusion of cells in the blastocoelic cavity was also observed. This anomaly is associated with reduced embryo viability in sheep and cattle (Steen Willadsen, personal communication), but its impact on human embryo viability is uncertain.

Other abnormal features in human blastocysts described in this and other studies have been associated with reduced total cell count, low or absent HCG secretion *in vitro* (Dokras *et al.*, 1993), and reduced implantation after intrauterine transfer (Jones *et al.*, 1998; Gardner *et al.*, 2000). Furthermore, total blastocyst cell count has been associated with hatching ability *in vitro* (Van Blerkom, 1993).

The appearance of a single cleavage anomaly on day 2 and/or 3 of development led to a significant reduction in normal blastocyst formation on day 5. Only 17% of embryos with >15% fragmentation formed a morphologically normal blastocyst. Such embryos have been shown to develop into fetuses at a higher rate after assisted hatching, fragment

removal, and uterine transfer on day 3 (Alikani *et al*., 1999). A further analysis for this study of 118 homogeneous transfers (all embryos transferred in one morphological category) involving embryos with >15% fragmentation revealed that ~18% implanted; on the other hand, the loss of ~80% of such embryos between day 3 and day 5 *in vitro* yields a theoretical day 5 implantation rate of ~8%. This suggests that the developmental potential of some of these embryos may be reduced in extended culture.

The 8% value seems to be in agreement with the ~5% implantation rate for fragmented embryos reported by a number of investigators (Staessen *et al*., 1993; Giorgetti *et al*., 1995; Ziebe *et al*., 1997). However, this low rate should be surprising, in view of the prevalence of fragmentation among in-vitro-generated embryos. Eighty per cent of all day 3 embryos in our database (40 438/49 801) had some degree of fragmentation. Also, fluorescence in-situ hybridization (FISH) analysis of embryos with ⩽35% fragmentation shows that about one-half are normal for the number of chromosomes tested (S.Munné, personal communication), and aneuploidy is not a major abnormality among such embryos (Munné *et al.*, 1995; Marquez *et al*., 2000). Instead, these embryos display different forms of mosaicism, some of which are compatible with normal development (reviewed by Munné and Cohen, 1998). Together, these findings suggest that fragmentation *per se* is not an abnormality. Moreover, with the exception of extreme cases where 40–100% of the embryonic volume has been lost and chromosomal abnormality is almost certain, it has been shown that the potential of moderately fragmented embryos for implantation may be determined by the distribution and size of the fragments (Alikani and Cohen, 1995; Alikani *et al*., 1999; Antczak and Van Blerkom, 1999). Here, reduced rates of normal compaction, cavitation, and blastocyst formation were observed in embryos with type IV fragments (large scattered fragments associated with one or more uneven cells) but not types I, II or III. If fragmentation results in the depletion of cortically positioned regulatory proteins essential to the embryo, as suggested by Antczak and Van Blerkom (1999), then type IV fragmented embryos may be specially affected, since the fragments are much larger than those in other types. These embryos had the lowest implantation rate after transfer on day 3 (Alikani *et al*., 1999) but extended culture may reduce their potential even further as less than a quarter may survive to the blastocyst stage (Table V).

We previously suggested that fragment removal may, at least in part, explain the high implantation rates we have reported for 6–35%, non-type IV fragmented embryos (Cohen *et al*., 1994; Alikani *et al*., 1999). Whether fragment removal on day 2 or 3 can lead to better blastocyst formation in-vitro remains to be clarified, but there is some firm indication that both cleavage between days 2 and 3 (Zaninovic *et al*., 1999) and compaction between days 3 and 4 (M.A., unpublished observation) are promoted if fragments are microsurgically removed.

Embryos that successfully underwent normal compaction, cavitation and ICM formation had significantly higher mean cell numbers on days 2 and 3 compared to those that failed to complete these developmental stages (Figure 4). Somewhat surprisingly, fast-cleaving embryos, particularly those with 9–10 cells on day 3, showed a reduced capacity to form normal blastocysts. In a study by Ziebe *et al*. (1997), the transfer of 4-cell embryos on day 2 resulted in a significantly higher pregnancy rate than the transfer of embryos beyond the 4-cell stage. Unusually fast-developing embryos may exhibit high levels of chromosomal aberration (Magli *et al*., 1998), some of which are due to polyspermic fertilization (Harper *et al*., 1994). These observations conflict with actual transfer results obtained in our Institute. In homogeneous transfers on day 3, the implantation rate of embryos with >9 cells was 32.4% (35/108) a high rate, albeit slightly lower than that of embryos with 7–9 cells 35.5% (1019/2874).

In a large study by Huisman *et al*. (1994), slow embryos showed lower implantation potential after 2, 3 or 4 days in culture. In the present study, prolonged culture of such embryos often led to abnormal patterns of compaction, specifically 'fusion-like' compaction, and abnormal cavitation (Figure 1). By contrast, homogeneous transfer of slow embryos on day 3 (between 4 and 5 cells) produced an implantation rate of 22%, suggesting either a normalization of development rate after intrauterine transfer of some slow embryos, or the receptivity of the uterus to late blastocysts developed in-vivo.

The reduced capacity of embryos with MNB to form blastocysts was demonstrated by Balakier and Cadesky (1997). The majority of the embryos in that study arrested at 2–15 cells and only 14% formed morphologically normal blastocysts. It has also been shown that multinucleation leads to low implantation rates after day 3 transfer (Jackson *et al*., 1998; Pelinck *et al*., 1998), as was observed in this study. Interestingly, multinucleation was the only cleavage anomaly that had a marked negative association with the development potential of embryos regardless of the duration of culture (Table V).

Many of the embryos with multinucleate blastomeres failed to show signs of compaction. When they did compact, their attempt at cavitation often ended with the persistence of what should be only transitory structures (e.g. intracellular vacuoles) involved in the formation of the blastocoel (Gualtieri *et al*., 1992). Embryos with MNB had a lower cell number (on average 6.5) on day 3, and formed blastocysts at a rate of 15.9%; this figure is not different for the rate at which all embryos with <7 cells formed blastocysts (13.8%). So, it is possibly both chromosomal anomalies associated with multinucleation (Kligman *et al*., 1996; Laverge *et al*., 1997; Staessen and Van Steirteghem, 1998) as well as their lower cell number that contribute to reduced blastocyst formation and reduced implantation in this group.

Under the conditions of this study, extended culture led to a reduction in viability of embryos with cleavage abnormalities. On this basis, and until further refinement of culture media, we suggest that extended culture should be limited to those embryos with optimal development during the first 3 days in culture. In the future, embryo selection based on oocyte and embryo polarity (Edwards and Beard, 1997), zygote morphology (Wright *et al*., 1990; Sadowy *et al*., 1998; Scott and Smith, 1998; Tesarik and Greco, 1999) and expression of developmental genes linked to implantation (Steuerwald *et al*.,

1999) may offer an alternative to extended culture and the associated loss of potentially viable embryos.

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