Article

Embryo culture as a diagnostic tool

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Abstract

Embryo culture can serve as a strong diagnostic tool, yielding useful information regarding the implantation potential of the human embryo. The information thus gained is useful for quality control of the embryology laboratory, success rates of the IVF/intracytoplasmic sperm injection (ICSI) programme, and counselling of the couple following failed cycles. Zygotes can be scored and zygote quality has been associated with further embryonic development and cleavage stage embryo quality. Early cleavage, cleavage rate, cleavage stage embryo grade and subsequent progression of these embryos to the blastocyst stage have all been shown to be individual and collective markers for the implantation-competent human embryo. This manuscript discusses embryonic markers of normality/quality throughout in-vitro culture starting with the zygote and ending with the blastocyst.

Keywords: *blastocyst, embryo, in-vitro culture, zygote*

Introduction

How can the implantation-competent human embryo be predicted? Embryologists and the clinicians have been searching for the answer to this intriguing question since the advent of assisted reproduction (Boiso, 2002). Before the era of controlled ovarian stimulation, decision-making was easy as there were no surplus embryos. The question that occupied minds then was why all human embryos did not implant. The latter still remains mainly unanswered, despite the passing of more than two decades since the first baby born through IVF. Not too soon, it was recognized that pregnancy rates in assisted reproduction could be increased with the transfer of more than one embryo. This led to the use of ovarian stimulating agents to make the ovaries produce more oocytes. More oocytes translated into more embryos available for transfer. Two inherent problems arose immediately; how to select the embryo that will implant and what to do with the surplus? There was an era where transfer policies were largely unrestrained. While liberal embryo transfer increased the success of assisted reproduction, at the same time it resulted in unacceptably high multiple pregnancy rates. Disadvantages and risks of multiple pregnancies were recognized immediately, leading to restriction of the number of embryos to be transferred in some countries. It became more important than ever to be able to choose the 'right embryo', the one that is destined to implant.

There are two main obstacles in human assisted reproduction that have to be overcome. One is the mechanism (molecular/cellular/genetic) and factors involved in the intricate process of implantation. The second is the selection of the embryo endowed with the potential to implant and yield a healthy offspring. This manuscript will deal mainly with the second question, and will try to shed light on embryo quality parameters that can be disclosed and perhaps altered during in-vitro culture (**Table 1**).

Embryology laboratory and its impact on embryo development and quality

Laboratory conditions are of utmost importance for preservation of the viability and the implantation potential of in-vitro generated embryos. An embryo that may become adversely affected by laboratory conditions may manifest its shortcoming sometimes only at the time of implantation. Therefore quality control in the human embryology laboratory should not only take into account parameters such as fertilization, cleavage, embryo grade, and blastocyst formation, but also the ultimate test for embryo viability that is implantation.

Air systems

The effect of laboratory ambient air on embryo development has attracted remarkably little attention. There is evidence that chemical air contamination may affect all stages of embryo development and subsequently have a major impact on implantation and pregnancy rates. Most IVF laboratories have sources of contamination that exceed the levels measured in homes, businesses and schools (Hall *et al*., 1998). Sources of these contaminants may have their origin outside of the laboratory, such as car and industrial emissions. Others may originate within the laboratory, and may be due to materials used in the facility such as compressed gas, cleaning and sterilizing agents, plastic and stored materials. Specific group of products have been described such as anaesthetic gases, refrigerants, cleaning agents, hydrocarbons, and aromatic compounds such as benzene and toluene. The majority of these compounds are highly oil soluble and can be removed by a mineral overlay. Laboratory procedures can be developed that restrict the use of alcohols and other volatile organic compounds. Existing laboratories can be retrofitted with pollution control devices, using chemical adsorption, oxidation and HEPA filtration. Two reports show improved implantation rates following implementation of changes to laboratory design and air filtration technology (Mayer *et al*., 1999; Racowsky *et al*., 2000). Addition of air filters to the incubators and a mineral oil overlay system are two inexpensive measures that can be adapted to the laboratories to increase success rates (A Gilligan, personal communication).

Incubators

Carefully calibrated and controlled CO_2 incubators are critical for successful IVF. Double (5% CO_2 in air) and more recently triple (5% CO_2, 5% O_2, 90% N_2) air supplies are used in the incubators. The level of oxygen to which the embryos are exposed *in vivo* is significantly below the 20% concentration that is present in the air. Several studies in different species show that reduced oxygen tension (5–7%) in the incubator results in enhanced embryo development. Human embryos cultured in a low O_2 environment produce blastocysts with significantly more cells than embryos cultured in a high oxygen environment (Gardner *et al*., 1999). To maintain correct pH (just under 7.5) in bicarbonate-buffered culture media, 5% CO_2 is required. Although most culture media work over a wide range of pH (7.2–7.4), it is preferable to ensure that pH does not exceed 7.4. To achieve this goal, it is therefore advisable to use a CO_2 concentration between 6 and 7%.

Light and temperature variations

There is very little data on the effect of embryology laboratory lighting and temperature on subsequent embryo development and quality. Regarding light, Barlow *et al*. (1992) exposed mature oocytes to different intensities of visible light prior to fertilization *in vitro*. Compared with controls cultured under identical conditions but protected from light, no significant difference in cleavage rate was noted. Transferred blastocysts implanted at a similar rate in both groups. Conversely, Fisher *et al*. noted statistically significant impaired cell proliferation when rabbit embryos were exposed to room temperature and light (Fischer *et al*., 1988). Temperature fluctuations may cause disruption of the cytoskeleton and have adverse effects on spindle integrity, resulting in chromosomally abnormal embryos (Almeida and Bolton, 1995). Transient cooling to room temperature caused reduction in spindle size, disorganization of microtubules within the spindle itself and sometimes complete lack of microtubules (Pickering *et al*., 1990). Strict temperature control therefore appears to be essential when handling gametes and embryos.

Table 1. Embryo characteristics that can be assessed during in-vitro culture.

Embryo characteristic	*Time of assessment*	*Correlation with implantation*
Pronuclear morphology	14–17 h post-insemination	Fair–good
Early cleavage	25–27 h post-insemination	Good
Cleavage rate	48–72 h post-insemination	Good
Cleavage stage embryo morphology	48–72 h post-insemination	Fair–good
Presence of freezable cleavage stage embryos	48–72 h post-insemination	Good
Progression to the blastocyst stage	120–144 h post-insemination	Good
Blastocyst quality	120–144 h post-insemination	Good

Embryo culture media

New and improved embryo culture media allow prolongation of the in-vitro culture period beyond that of the cleavage stage. In the early days of IVF, embryos were cultured for up to 48 h following oocyte retrieval. In the 1990s, co-cultures using various feeder cells enabled embryo culture up to 5–6 days after oocyte retrieval. However, co-cultures were cumbersome and susceptible to inter-laboratory variations that prevented replication of results. Sequential media that took into account the variation of nutritional needs of the embryo at different stages of development allowed better yield in terms of blastocyst generation from cleavage stage embryos that were cultured *in vitro* for extended periods of time. Accessibility and the range of reportedly successful media products have resulted in an exponential rise in the acceptability of prolonged in-vitro culture and late embryo transfer. Approximately one-third to two-thirds of the embryos cultured in stage specific media have been shown to progress to the blastocyst stage. When sequential media are used, lactate and non-essential amino acids are included for culturing cleavage stage embryos. At this stage, the media contains pyruvate but no glucose, which is considered toxic for embryos aged 1–3 days. Subsequently for blastocyst culture, glucose, essential and non-essential amino acids, vitamins and nucleic acid precursors are added (Gardner and Lane, 1997).

New culture media were designed taking into account the environment to which the embryos are exposed *in vivo* and also studying the physiology and metabolism of the embryo in culture to determine what causes intracellular stress to the developing embryo (Lane and Gardner, 2000, 2002). The key to successful culture of mammalian embryos is to minimize intracellular stress (Gardner, 2003). Stress can be induced by inappropriate media formulations. It is interesting to note in the literature extreme differences in blastocyst formation and pregnancy rates with blastocyst transfer. Amino acids in sequential or complex media may release ammonium ions into the culture system and cause retardation of embryonic and fetal growth (Lane and Gardner, 1994). To prevent ammonium-induced embryonic damage, the culture medium should be renewed every 48 h and the major source of ammonium, glutamine, should be replaced by alanyl-glutamine (Gardner, 2003). Inclusion of serum in the culture systems may have serious detrimental effects on embryo physiology, metabolism, gene expression and embryonic growth. Therefore it is advisable not to use serum in current culture systems.

Embryos that are cultured *in vitro* allow several parameters of viability and normality to be observed and assessed by the laboratory personnel. These may be grouped into: (i) fertilization and its abnormalities; cytogenetics of abnormal fertilization, (ii) assessment of pronuclear morphology, (iii) early cleavage, cleavage rate, and cleavage stage embryo morphology, and (iv) blastocyst formation

Fertilization and abnormalities

Following IVF, inseminated oocytes must be dissected and the surrounding cumulus cells be removed prior to assessment of fertilization. Oocytes that were fertilized through intracytoplasmic sperm injection (ICSI) have already had all cells removed prior to injection and thus can be directly assessed for fertilization without further treatment. Visualization of two pronuclei indicates normal fertilization. Should pronuclear scoring be performed, this should be undertaken 17–20 h after insemination, before the pronuclei merge during syngamy.

Normally fertilized oocytes should have two pronuclei, two polar bodies, regular shape with intact zona pellucida and a clear healthy cytoplasm. The number, size, and the position of pronuclei as well as the nucleolar precursor bodies have been shown to correlate with developmental potential of the zygotes and this may be related to polarization within the oocyte and eventually the zygote. Cytoplasm of normally fertilized oocytes tends to be slightly granular, whereas the cytoplasm of unfertilized oocytes tends to be completely clear and featureless. There may be different shaped abnormalities, and these do appear to affect normal embryo development.

In-vitro development potential of zygotes with uneven pronuclei was examined by Sadowy *et al*. (1998). The authors concluded that zygotes with uneven pronuclear size arrested more frequently at the 1-cell stage and gave rise to embryos with increased multinucleation on day 2. Furthermore, mosaicism was more common in these embryos when analysed by fluorescence in-situ hybridization (FISH) on day 3.

Oocytes with a single pronucleus represent approximately 3–4% of all in-vitro-fertilized oocytes. They may result from parthenogenetic activation of the oocyte, from asynchrony in pronuclei formation (retarded appearance of the second pronucleus) of from precocious syngamy. As the above suggest, zygotes with single pronuclei are not always abnormal. Such zygotes that were obtained after IVF were analysed by FISH to determine their ploidy status. Of the 16 zygotes analysed as such, 10 were haploid and six were diploid. It seems that during the course of their interaction, it is possible for human gamete pronuclei to associate together and form diploid, single pronucleate zygotes. These findings confirm a newly recognized variation of human pronuclear interaction during syngamy and single pronucleate zygotes that develop normally *in vitro* can be replaced (Levron *et al*., 1995).

In a follow-up study, 42 day 3 embryos that developed from 1PN zygotes were analysed by FISH (Sultan *et al*., 1995). In patients who had undergone conventional insemination by IVF, 62% of the embryos developing from 1PN zygotes were diploid and nearly 50% contained a Y chromosome, indicating fertilization by a spermatozoon. However, only 14% of the 1PN zygotes obtained after ICSI were diploid and only 19% contained a Y chromosome. Based on the findings of this follow-up study it appears prudent to replace 1PN zygotes from conventional IVF cycles. Caution is recommended, however, prior to considering transfer of embryos derived from 1PN zygotes derived from ICSI.

Zygotes showing more than two pronuclei should be definitely considered as abnormal and cultured separately. Tripronuclear zygotes represent approximately 5% of the embryos fertilized *in vitro* and may arise from fertilization of a diploid oocyte by a single spermatozoon (digynic triploids), or fertilization of a normal egg by two spermatozoa (diandric triploids).

Tripronuclear embryos should not be selected for transfer. It is important to recognize these immediately, as once cleavage occurs they will be indistinguishable from normal zygotes.

Triploidy has been reported in 1% of all conceptions. Dispermy accounts for two-thirds of all triploid pregnancies, while the remainder is due to diploid oocytes that have failed to complete oogenesis. Benkhalifa and co-workers (1996) studied normal looking zygotes that have failed to undergo cleavage division. Approximately half of these zygotes' oocyte chromosomes with two chromatids each, while spermatozoa chromosomes were in the form of single chromatids. This makes it likely that failure to complete the second stage of meiosis by the oocyte chromosomes is directly responsible for arrest of embryonic development at the zygote stage (Benkhalifa *et al*., 1996). Failure of oocyte activation or structural defects or incorrect location of the sperm centrosome is responsible for most of the remainder of pronuclear stage cleavage arrest (Van Blerkom *et al*., 1995).

Assessment of pronuclear morphology

Assessment of the zygote and the cleavage stage embryo is a crude science. This makes it difficult for the clinician to select the most appropriate embryo/s for transfer. Tesarik and Greco defined a set of criteria for pronuclear morphology and related this to other parameters of embryo normality or quality, such as the occurrence of cleavage arrest, the incidence of blastomere multinucleation and cleavage stage embryo morphology (Tesarik and Greco, 1999). In this study, they showed that when compared in the whole unselected group of embryos, those arising from pattern 0 zygotes (ideal pronuclear pattern) resulted in better quality cleavage stage embryos and were less likely to arrest at the cleavage stage. This assessment system may enable the embryologist to select the embryo with the highest implantation potential as early as possible. Furthermore, ethical concerns regarding selection of embryos for fresh transfer versus cryopreservation may be obviated.

Scoring of zygotes according to Tesarik and Greco is summarized as follows: 0PN pattern: the number of nucleolar precursor bodies (NPB) in both pronuclei never differs by more than three; NPB polarized when fewer than seven and never polarized when more than seven in at least one pronucleus; the number of NPB in a pronucleus never fewer than three; the distribution of NPB either polarized or non-polarized in both pronuclei. Zygotes that did not conform to this morphological pattern were considered as abnormal and classified into one of the five following patterns: pattern 1 included zygotes that presented a large difference (>3) in the number of NPB in both pronuclei; pattern 2 included zygotes with a small number of (<7) of NPB with polarization in at least one pronucleus; pattern 3 included zygotes with a large number (>7) of NPB with polarization in at least one pronucleus; pattern 4 included zygotes that had a very small number of (<3) NPB in at least one pronucleus; and pattern 5 presented a polarized distribution of NPB in one pronucleus and non-polarized in the other (Tesarik and Greco, 1999).

Several other criteria of embryo quality such as early cleavage, strict cleavage stage embryo morphology assessment, and the presence of 8-cell embryos on day 3 may be combined with pronuclear morphology to make a more accurate selection. Correlation between pronuclear morphology and other reported markers of embryo normality have been recently studied. Wittemer *et al*. (2000) studied embryo development to day three and correlated different embryological parameters with pronuclear scoring. They concluded that pattern 0 zygotes lead to more good quality embryos with a higher implantation potential than embryos developing from less favourable zygote patterns.

Scott *et al*. used a previously defined pronuclear scoring system revised during their study and calculated a zygote score based on zygote grade, day 3 embryo morphology and the ability of the embryos to reach the blastocyst stage (Scott *et al*., 2000; Scott, 2003). Cleaving embryos that were selected initially by zygote morphology and secondarily by embryo morphology on day 3 implanted at a higher rate compared with those selected by morphology alone (31 versus 19%). Zygote scored blastocyst transfer cycles resulted in higher implantation rates compared with non-scored cycles (58 versus 39%). The authors concluded that zygote scoring increased implantation and pregnancy rates both on day 3 and day 5. Zygote scoring showed a strong correlation between both embryo morphology on day 3 and the ability of the individual embryo to reach the blastocyst stage on day 5 or 6.

Ludwig *et al*. (2000) defined a pronuclear score where they graded zygotes 16–18 h post-ICSI according to the position of the pronuclei, the alignment of the nucleoli at the junction of the two pronuclei, and the appearance of the cytoplasm. In this study, embryo selection was performed solely by pronuclear scoring. A threshold value of 13 for zygote score was accepted as a cut-off, as this appeared to allow prediction of the establishment of pregnancy. Cumulative embryo score on the day of transfer was similar in patients with zygote scores of >13 and <13. Pronuclear score did not differ in those cycles with and without male factor. Pregnancy rates were 4 and 22% when the PN score was <13 and >13 respectively.

A report has been published recently on the embryo quality parameters such as early cleavage, cleavage stage embryo morphology and cleavage rate, and progression to the blastocyst stage and their correlation with pronuclear morphology (Balaban *et al*., 2001b). The results of this study are in line with other studies in the literature, and suggest that there is a good relationship between markers of embryo normality/quality starting from the zygote stage up to the blastocyst stage (**Table 2**). Zygotes showing an ideal pronuclear pattern end up as embryos that cleave earlier and faster, that have better cleavage stage morphology, reach the blastocyst stage more often and that are of better grade blastocysts. Furthermore, blastocysts arising from zygotes with an ideal pronuclear pattern implant more efficiently resulting in higher clinical pregnancy rates. Grade 1 and 2 blastocysts derived from 0 pattern zygotes had the highest implantation potential. This is the group that may be a candidate for elective single embryo transfer (eSET). Grade 1 and 2 blastocysts derived from zygotes with abnormal PN patterns also implanted, albeit at a lower rate.

Table 2. Relationship between pronuclear morphology, cleavage rate, blastocyst progression, blastocyst grade, and multinucleated blastomeres (from Balaban *et al*., 2001b).

PN pattern	Equal-sized 2PN	Early cleavage	>8 cell embryos on day 3	Blastocyst formation	BG1 + BG2 blastocysts	Hatching blastocysts	% MNB[a]
Ideal PN pattern (%)	109 (16.1)	27 (25.2)	58 (54.2)	77 (71.9)	61 (79.2)	22 (28.5)	4 (3.7)
Single PN anomaly (%)	477 (70.5)	39 (8.6)	139 (30.9)	160 (35.7)	79 (49.3)	19 (11.8)	59 (13.1)
Double PN anomaly (%)	90 (13.4)	3 (4.5)	11 (16.7)	15 (12.7)	7 (46.7)	1 (6.7)	8 (12.1)

[a]MNB = multinucleated blastomeres.

Lundqvist retrospectively analysed 340 patients who underwent IVF where pronuclear morphology and early cleavage were recorded. Embryo transfers were based on cleavage stage embryo morphology. Significantly more embryos implanted when pronuclear morphology was favourable (24.6 versus 19.1%) ($P < 0.05$) (Lundqvist, 2000).

It appears from the analysis of results of several studies in the literature that PN scoring can successfully be incorporated into the practice of embryo evaluation for prediction of implantation potential of the individual embryo. Markers of embryo normality so far defined appear to have a strong relationship with each other. Studies indicate that the 'good' embryo can be determined at the onset of embryonic development as early as the zygote stage. Embryos preselected for transfer at this stage appear to be the embryos that cleave faster, have better cleavage stage morphology, reach the blastocyst stage more often and give rise to better quality and hatching blastocysts. Nucleolar patterns are closely linked to embryo viability, reinforcing the relevance of polarity in human oocytes and embryos (Edwards and Beard, 1997).

Early cleavage, cleavage rate, and cleavage stage embryo morphology

Early cleavage, cleavage rate, and cleavage stage embryo morphology, cytoplasmic fragmentation and multinucleation have been shown to be important markers of embryo quality and viability. These features of the embryo may be observed over time during in-vitro culture.

Early cleavage

There is reliable data in the literature suggesting that early cleavage is a strong marker of embryo quality and viability. Early cleavage is defined as cleavage occurring 25–27 h after insemination. Shoukir *et al*. (1997) showed that embryos cleaving to the 2-cell stage 25 h post-insemination in conventional IVF yielded higher pregnancy rate upon transfer. Furthermore, early cleaving embryos were of higher quality. The same group reported similar findings also with ICSI embryos (Sakkas *et al*., 1998). In this later study, embryos from 45% of the women showed early cleavage. As the number of early cleaving embryos in the transfer cohort increased from zero to two, there was a parallel increase in the implantation and pregnancy rates. Platteau *et al*. (1999) showed higher implantation and pregnancy rates with the transfer of early cleaving embryos. Early cleaving embryos also reached the blastocyst stage at a higher rate. In a more recent study, Isiklar *et al*. retrospectively evaluated 78 ICSI–embryo transfer cycles where 48 cycles included at least one early cleaving embryo in the transfer cohort, whereas 30 had no cleaving embryos available for transfer (Isiklar *et al*., 2002). Clinical pregnancy and implantation rates were 17.9 versus 5.1% and 45.8 versus 16.7% respectively in the early cleavage and no early cleavage groups. Of the excess embryos, 56.7% of the early cleaving ones reached the blastocyst stage compared with 25% of the non-cleaving ones (**Table 3**). More recently, Salumets *et al*. in elective single embryo transfer cycles showed that early cleaving embryos upon transfer

Table 3. Presence of early cleavage and outcome of ICSI (Isiklar *et al*., 2002).

Characteristic	*No early cleavage*	*Early cleavage*	P-*value*
No. of cycles	30	48	–
Mean age (years)	34.2	33.1	NS
No. oocytes	11.9	12.8	NS
Fertilization rate (%)	74.8	77.3	NS
No. embryos transferred	3.9	4.0	NS
Implantation rate (%)	5.1	17.9	<0.05
Clinical pregnancy/embryo transfer (*n*)	16.7	45.8	<0.05
Blastocyst progression of excess embryos (%)	25.0	56.7	<0.05

implanted at a higher rate compared with embryos showing no early cleavage (Salumets *et al*., 2003). This is an important study, as all previous studies included in the transfer cohort early cleaving embryos as well as embryos that showed no early cleavage. The authors based their embryo selection criteria on embryo morphology on day 2, disregarding early cleavage status of that particular embryo. However, upon analysis of results it became evident that embryos with similar morphology on day 2 differed in their implantation potential. Embryos showing early cleavage and four blastomeres on day 2 implanted at a higher rate compared with embryos showing no early cleavage but similar number of blastomeres. When the authors analysed the factors predicting clinical pregnancy, only the percentage of evenly cleaved embryos and early cleavage were found to be significant.

Cleavage rate

It has been previously shown for day 2 embryos that cleavage to the 4-cell stage resulted in higher implantation efficiency (Giorgetti *et al*., 1995; Ziebe *et al*., 1997). A retrospective analysis of day 2 and 3 transfers, showed that embryos that had at least four or five blastomeres on day 2 and at least seven blastomeres on day 3 yielded higher implantation rates (Gerris *et al*., 1999; Van Royen *et al*., 1999, 2001). Employing these criteria, Gerris *et al*. selectively transferred a single embryo to 26 patients <34 years of age and obtained an ongoing pregnancy rate of 38.5%. The implantation rate was 42.3% and there were no twin pregnancies (Gerris *et al*., 1999). Van Royen *et al*. (1999), in a step towards single embryo transfer tried to characterize embryos with the highest implantation potential. They retrospectively analysed all twin pregnancies resulting from the transfer of two embryos. Embryos with no multinucleated blastomeres, four of five blastomeres on day 2 and seven or more blastomeres on day 3, and <20% anucleate fragments were associated with the highest implantation potential.

Slow as well as fast cleaving embryos implant less efficiently. There is surprisingly very little data on the implantation potential of slow-cleaving embryos. In mice, lack of a specific protein product of *preimplantation development* (*ped*) gene leads to slower developing embryos that implant as frequently as their normal developing counterparts but are aborted in mid-gestation (Warner *et al*., 1998). A human homologue of this gene probably exists in the human (Cao *et al*., 1999). Due to lack of data regarding the normality and fate of slow cleaving embryos, these are currently considered for transfer. However, lower implantation rates and blastocyst formation should be expected of such embryos (Alikani *et al*., 2000). The incidence of 8-cell embryos on day 3 appears to be correlated closely with PN pattern at the zygote stage (Balaban *et al*., 2001a). While 54% of the of the zygotes with ideal PN pattern had >8 blastomeres on day 3, only 17% of the zygotes having double PN abnormality resulted in cleavage stage embryos with >8 blastomeres.

Similar to slow cleaving embryos, fast cleaving embryos have also been considered as abnormal. Fast embryo cleavage has been linked to high levels of mosaicism and polyspermic fertilization (Harper *et al*., 1994). Furthermore, over 70% of fast cleaving embryos were found to be chromosomally abnormal (Magli *et al*., 1998).

It is suggested that on day 3 of in-vitro culture, available embryos showing 7–8 blastomeres should be considered for transfer and embryos having fewer or more blastomeres for cryopreservation.

Cleavage stage embryo morphology

Assessment is usually undertaken on days 2 or 3 of embryonic development. Blastomere size/symmetry and the degree of fragmentation are taken into account when cleavage stage morphology is assessed. Cleavage stage embryos are graded as follows: grade 1 embryo, no fragmentation with equal sized homogenous blastomeres; grade 2 embryo, <20% fragmentation with equal sized homogenous blastomeres; grade 3 embryo, 20–50% fragmentation with unequal sized blastomeres; grade 4 embryo, >50% fragmentation with unequal sized blastomeres. Currently used morphological features in embryo grading include cell number, blastomere symmetry, and the degree of fragmentation and were described for day 2 embryos (Puissant *et al*., 1987; Staessen *et al*., 1992; Steer *et al*., 1992; Ziebe *et al*., 1997). For embryos cultured until day 3, inclusion of other parameters such as fragmentation pattern, cytoplasmic pitting, compaction, blastomere expansion and absence of vacuoles appears to increase the predictive value of implantation potential of the selected embryo (Desai *et al*., 2000).

Embryos with asymmetric blastomeres should be considered subnormal and excluded from transfer when possible. These embryos show a higher degree of multinucleation and aneuploidy (Hardarson *et al*., 2001). Alikani *et al*. (1999) showed that the degree and pattern of fragmentation significantly impacted pregnancy and implantation. The same group, in a more recent study examined the relationship between morphological anomalies of cleavage stage embryos and their ability to form normal blastocysts *in vitro* (Alikani *et al*., 2000). A normal cleavage rate (7–9 cells on day 3) was associated with a higher progression rate to the blastocyst stage, whereas excessive embryo fragmentation (>15%) had a negative impact. Besides the degree of fragmentation, pattern of fragmentation also appears to be important. Larger fragments that are associated with uneven blastomeres appear to be the most detrimental. Low implantation rates should be expected from the transfer of such embryos. Antczak and Van Blerkom (1999) recently showed that fragmentation results in partial or total loss of regulatory proteins form affected blastomeres affecting subsequent development of the embryo. Measures such as fragment removal have been proposed to salvage embryos showing extensive fragmentation (Alikani *et al*., 1999). Whether these measures are of any value, however, remains to be determined.

Embryos with multinucleated blastomeres also should be considered abnormal and not transferred. Follicular underoxygenation and rapid follicular growth associated with a very short follicular phase have been proposed as possible mechanisms for multinucleation (Van Blerkom *et al*., 1997). Multinucleation can result from karyokinesis without cytokinesis, fragmentation of nuclei, or abnormal migration of chromosomes during anaphase (Tesarik *et al*., 1987; Hardy *et al*., 1993). Kligman showed using FISH analysis that multinucleated embryos had extensive mosaicism or polyploidy (Kligman *et al*., 1996). The incidence of genetic

abnormalities was lower, however, when multinucleation occurred on day 4 or later of embryonic development (Munné and Cohen, 1993; Munné *et al*., 1994). It is generally advised that multinucleated embryos at the 2-cell stage should not be transferred; however, embryos showing multinucleation at or beyond the 8-cell stage may be considered for transfer. Multinucleation has also been linked to excessively high oestradiol levels and large number of retrieved oocytes (Jackson *et al*., 1998; Styne *et al*., 2001). Stimulation protocol seemed to have no effect.

The impact of multinucleated blastomeres on the developmental potential of embryos up to the blastocyst stage has recently been evaluated (unpublished data). Of the 7982 embryos evaluated retrospectively from 619 ICSI cycles, 1568 (19.6%) showed multinucleated blastomeres on day 2 or 3 of development. These embryos were not considered for transfer and were cultured up to the blastocyst stage. While the cleavage rate of embryos without multinucleated blastomeres was 92%, this declined to 56 and 39% respectively when the embryo contained one or more blastomeres with multinucleation. Furthermore, the rate of blastocyst formation declined from 51% to 6.4 and 2.4% when the embryos showed no multinucleation or multinucleation in one or more blastomeres. None of the blastcysts derived from embryos with multinucleation was of good quality.

Racowsky (2003) assessed the morphology viability predictors in day 3 embryos. Cleavage rates significantly declined with advancing age and the performance of ICSI. Patients who were 37 years or older had more embryos with fewer than 4 cells and fewer embryos with more than 8 cells. When embryo viability parameters were studied individually: embryos with 8 cells on day 3, embryos with no fragmentation or <10% fragmentation, and embryos with even blastomeres had the highest viability.

Blastocyst formation

Embryo transfer is performed 2, 3 or 5 days after oocyte retrieval. Prolonging the culture period allows for better selection of more advanced embryos that are not arresting, as laboratory assessment is undertaken after the expression of the embryonic genome. At this stage, paternal genetic factors that influence embryo viability have also made their impact that may allow selection of the genetically normal embryo. Advantages of blastocyst stage embryo transfer include better synchronization between the endometrium and the embryo and possible selection of embryos with higher implantation potential (Langley *et al*., 2001; Karaki *et al*., 2002; Kolibianakis, 2002; Wilson *et al*., 2002). In the embryology laboratory, blastocyst culture may be regarded as a quality control measure. Furthermore, in programmes dealing with preimplantation genetic diagnosis, blastocyst culture is a necessity.

Whether characteristics of the zygote and the cleavage stage embryo is associated with subsequent blastocyst formation is important, as if there is a close correlation with early embryonic markers of normality, blastocyst culture may be obviated. There are several studies that address this question. Balaban *et al*. (2000), in 350 day 5 embryo transfer cycles, showed a close correlation between cleavage stage embryo grade and subsequent progression to the blastocyst stage. While 59.1% of grade 1 and 2 cleavage stage embryos progressed to the blastocyst stage, only 25.9% of grade 3 and 4 embryos showed similar progression (**Table 4**). However, when blastocyst stage was reached, blastocyst quality was not affected by cleavage stage embryo morphology. Poor quality cleavage stage embryos formed good quality blastocysts at a similar rate compared with good quality cleavage stage embryos.

Shapiro and co-workers (2000) studied the predictive value of day 3 blastomere number on subsequent blastocyst development. More blastomeres on day 3 were associated with higher rate of progression to the blastocyst stage. While only 32% of 6-cell embryos on day 3 reached blastocyst stage, similar rate was 75% for 8-cell embryos. However, 25% of the fastest growing embryos still did not reach the blastocyst stage.

Graham and co-workers showed that only 48% of the embryos selected on day 3 for transfer or cryopreservation were reselected on day 5 for transfer (Graham *et al*., 2000). Rjinders and Jansen also reported that 51% of the embryos transferred on day 5 had been selected for transfer on day 3 (Rjinders and Jansen, 1998).

In an attempt to correlate markers of embryonic normality with subsequent blastocyst development, Balaban *et al*. (2000) showed that embryos with ideal pronuclear morphology, good cleavage stage morphology and having at least 8 blastomeres on day 3 of development progressed to the blastocyst stage at a higher rate and formed better quality blastocysts.

Blastocyst quality has been associated with the success of blastocyst transfer. Blastocysts may be graded according to the criteria proposed by Dokras and co-workers or by Gardner and Schoolcraft (Dokras *et al*., 1993; Gardner and Schoolcraft, 1999). Dokras and co-workers used a development rate and morphology based scoring system and furthermore correlated blastocyst morphology and total cell count with human chorionic gonadotrophin (HCG) secretion *in vitro*. Blastocysts were graded as BG1, BG2, and BG3. BG1 referred to early

Table 4. Relationship between embryo quality on day 3 and blastocyst quality on day 5 (from Balaban *et al*., 2000).

	Grade 1 + 2 day 3 embryos	*Grade 3 + 4 day 3 embryos*	P-*value*
No. of embryos	2293	1220	NS
Blastocysts (%)	59.1	25.9	<0.05
G1 + G2 blastocysts (%)	59.8	56.1	NS
G3 blastocysts (%)	40.2	43.9	NS

cavitation, resulting in the formation of eccentric and then expanded cavity lined by a distinct inner cell mass and trophoectoderm on day 5. BG2 blastocysts were morphologically similar to BG1 blastocysts, but with a 1- or 2-day delay in the visible differentiation of the two cell types. Vacuolated embryos as well as blastocysts with several degenerative foci in the inner cell mass and collapse of the cavity without re-expansion were graded as BG3. In the Gardner grading system, initially blastocysts are given a numerical score from 1 to 6 based on their degree of expansion and hatching status. Inner cell mass and trophoectoderm grading is then assessed. The two grading systems were compared by Balaban *et al*. (2002). Blastocyst grading by both Dokras and Gardner appear to predict the success of blastocyst transfer relatively accurately. Gardner grading system appears to be a better predictor of pregnancy and multiple pregnancy rates especially when >3 AA blastocysts are transferred. Given the very high implantation rate in this group, single blastocyst transfer should be seriously considered. The Dokras grading system appears to be a better predictor of failure of blastocyst transfer. When only BG3 blastocysts are available the number to be transferred can be safely increased. However, this is still associated with a very low implantation rate per embryo (**Table 5**).

Transfer of embryos at later stages of development has been associated with higher implantation and pregnancy rates in some but not all studies (Scholtes and Zeilmaker, 1996; Coskun *et al*., 2000; Huisman *et al*., 2000; Karaki *et al*., 2002; Levron *et al*., 2002; Milki *et al*., 2002; Rienzi *et al*., 2002; Utsunomiya *et al*., 2002). It appears that the selection of the embryo endowed with the potential to implant is the crucial factor leading to success of late embryo transfer. Most of the reported studies dealt with highly selected patient populations thus making it difficult to assess the real value of blastocyst transfer in a general IVF/ICSI population. Randomized studies that deal with an unselected patient population suffered from inappropriate randomization protocols, use of a single medium for day 3 as well as day 5 transfers or use of several different sequential media during the study period (Scholtes and Zeilmaker, 1996; Utsunomiya *et al*., 2002). Gerris and co-workers analysed patients <34 years of age undergoing transfer of two top quality embryos on day 3 pregnancy and implantation rates of 74 and 48% were achieved. However, when the authors extended their observations to an unselected patient population rates for pregnancy and implantation were 49.6 and 39.5% (Gerris *et al*., 1999, 2001). At least for day 3 embryo transfers, this underlies the importance of patient selection. It is reasonable to assume that the same holds true for blastocyst transfers.

A recent prospective randomized study evaluated the outcome of day 2 transfer with that of blastocyst transfer in an unselected IVF/ICSI population (Van der Auwera *et al*., 2002). A total of 129 patients completed the study, of whom 63 received a mean of 1.9 embryos on day 2 and 66 received a mean of 1.9 blastocysts on day 5. Embryos were cultured in complex sequential media in both groups. Clinical pregnancy rate per embryo transfer (35 versus 60%) and delivery rates (30 versus 50%) were significantly increased in the blastocyst transfer group. It is of note that in this unselected patient population, 26% of the patients in the blastocyst transfer group failed to receive embryos.

Blastocyst transfer has also been advocated for patients with repeated day 2–3 embryo transfer failures. Simon *et al*. (1999) co-cultured embryos from oocyte donation patients up to the blastocyst stage. All patients had previous failed cycles. The implantation and pregnancy rates 32.7 and 54.5% in co-culture day 5 transfer cycles compared with 4.5 and 13.3 in previous day 2 transfer cycles. Success rates were decreased in patients who were transferred their own co-cultured blastocysts. In a recent study, Shapiro *et al*. (2001) showed very low implantation rates in patients who underwent unsuccessful blastocyst transfer attempts. These studies suggest that merely employing blastocyst transfer in couples with recurrent implantation failure does not improve success rates.

It has been speculated that embryos reaching the blastocyst stage are generally chromosomally normal and culturing embryos up to the blastocyst stage eliminates chromosomally abnormal embryos. It was shown that 54.4% of chromosomally normal embryos reached the blastocyst stage, compared with 33.6% of chromosomally abnormal embryos (Rubio *et al*., 2000). Therefore, blastocyst transfer does not universally select against abnormal embryos and cannot serve as a reliable marker of embryonic normality. In accordance with these results, Sandalinas showed that 19% of aneuploid embryos were able to reach the blastocyst stage (Sandalinas *et al*., 2001). Of the aneuploid embryos, 9% of monosomics and 37% of trisomics were able to form blastocysts.

It is important to select the cohort of embryos that is suitable for extended in-vitro culture. It appears that the whole cohort behaves similarly that is if embryos that are not selected for transfer progress to the blastocyst stage very high pregnancy rates are to be expected from their counterparts that are transferred on day 3 (Balaban *et al*., 1998). Blastomere number on day 3 appears to be a good indicator of subsequent blastocyst formation and implantation. In patients having no 8-cell embryos, blastocyst culture and transfer has been

Table 5. Blastocyst quality and outcome of blastocyst transfer: results from embryo transfer cycles with homogenous transfers (from Balaban *et al*., 2000).

	Transfer cycles with only grade 1 blastocyst transfers	*Transfer cycles with only grade 2 blastocyst*	*Transfer cycles with only grade 3 blastocysts*
No. of cycles	32	47	98
Clinical pregnancy/ embryo transfer (%)	68.7	61.7	13.3
Implantation rate (%)	56.2	46.4	7.1

associated with 0% pregnancy rate (Racowsky *et al*., 2000). This suggests that the uterine environment may rescue some of the embryos that will not implant as blastocysts. Balaban and co-workers (2001a) studied the impact of culturing grade 3 and 4 embryos up to the blastocyst stage to observe in-vitro development of these poor prognosis embryos. The study group was compared with a control group of patients who had exclusively poor morphology embryos that were transferred on day 3. Transfer of poor quality cleavage stage embryos after culturing them *in vitro* up to the blastocyst stage was associated with higher implantation rates.

In summary, blastocyst transfer enables the embryologist to: (i) select the most implantation-competent embryo among the cohort of transferable embryos, thus increasing implantation and decreasing multiple pregnancy rates; (ii) select against some of the genetically abnormal embryos; (iii) perform preimplantation genetic diagnosis; and (iv) test the laboratory's culture conditions.

Despite purported advantages of blastocyst culture and transfer, several concerns have been voiced, including interlaboratory variation of blastocyst formation, less than optimal results obtained with blastocyst crypreservation, and lack of available blastocysts for transfer, especially in women with few oocytes. Blastocyst culture has also been considered as an extra burden on the embryology laboratory personnel. More work time and more incubators need to be allocated when blastocyst transfer is an integral part of an assisted reproduction centre.

Embryos are exposed to more stress as the culture period is increased. Appropriate and well-designed culture systems are of profound importance prior to embarking upon blastocyst culture. The number of incubators should be increased, as blastocyst development and cell numbers significantly decreased when the incubator door was opened many times during the culture period (Gardner and Lane, 1996).

Damage to the embryo regarding metabolism and gene expression is manifest during the first three cleavage divisions, and not by blastocyst culture. Precompacted embryos are more vulnerable to culture-induced stress than an embryo that has formed a transporting epithelium at compaction (Edwards *et al*., 1998). Embryo culture in suboptimal conditions may be associated with altered gene expression and in some cases loss of imprinting (Ho *et al*., 1995; Doherty *et al*., 2000). Major epigenetic events take place during both germ cell development and preimplantation stages when assisted reproductive procedures are being carried out, possibly interfering with the proper establishment (in gamete culture) and maintenance (in embryo culture) of genomic imprints (Gosden *et al*., 2003). Recent reports of increased incidence of imprinting disorders such as Beckwith–Widemann syndrome and Angelman syndromes after assisted reproduction point out to the need for molecular characterization of epigenetic abnormalities, including the methylation status of imprinting control regions within imprinted gene clusters.

Preimplantation genetic diagnosis

Improved embryo culture conditions have allowed the embryo to survive following blastomere biopsy for preimplantation genetic diagnosis. Preimplantation genetic diagnosis can be used for aneuploidy screening in women with advanced age or recurrent implantation failure with IVF (Gianaroli *et al*., 2001; Pehlivan *et al*., 2002). Moreover, screening for translocations and single gene defects are also possible. Current methods allow screening for a certain number of chromosomes with multicolour FISH techniques. Comparative genomic hybridization and DNA microarrays will probably replace the current methods, as with these a full chromosome count is feasible. In case of aneuploidy screening transfer is affected after FISH results are available to the clinician. Without extended embryo culture, it would have not been possible to transfer the biopsied normal embryos in the same cycle. It has been proposed that extended culture would select against chromosomally abnormal embryos thus obviating the need to do embryo biopsy and subsequent FISH analysis. Culturing embryos up to the blastocyst stage may result in genetically defective embryos ceasing their development and allow normal embryos to survive for longer periods *in vitro*. Although monosomic and polyploidy embryos reached the blastocyst stage at a significantly lower rate, trisomic embryos compatible with fetal development are associated with relatively high rates of blastocyst formation (Márquez *et al*., 2000; Sandalinas *et al*., 2001). Furthermore, unbalanced embryos from translocation carriers also reach the blastocyst stage and implant as efficiently as normal embryos (Menezo *et al*., 1997; Evsikov *et al*., 2000).

Hardarson *et al*. studied morphological and chromosomal characteristics of embryos that were considered to be not suitable for transfer according to morphological criteria (Hardarson *et al*., 2003). All discarded embryos were observed until the blastocyst stage. Close to half of the surplus pre-embryos that reached the blastocyst stage were chromosomally normal when assessed with multicolour FISH. This study is important, as it shows that a significant proportion of the discarded embryos reach the blastocyst stage when cultured *in vitro*, and furthermore, a significant proportion of these are chromosomally normal. Results of this study should be considered when selecting embryos for cryopreservation as some of the embryos that are thought to be unsuitable actually carry the potential to implant.

It may be concluded that although prolonged in-vitro culture may select against certain types of chromosome abnormalities in human embryos, it cannot be considered as an absolute reliable tool. Preimplantation genetic diagnosis remains to be the gold standard for screening for abnormal chromosome constitution of the embryo.

Conclusions

In-vitro culture of preimplantation embryos yields very important information regarding embryo quality and normality. Couples who do not conceive can be counselled regarding their chances in subsequent attempts. Embryos demonstrate quality markers from the zygote up to the blastocyst stage. This enables the embryologist to select the most implantation competent human embryo. This will lead

the way to the not so elusive goal of transferring a single embryo, thus obviating the complications associated with multiple pregnancies. In the not so distant future, even better culture media and culture systems such as microfluidic channels will open new and exciting avenues in laboratory aspects of assisted reproduction.

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