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Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: a review

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In contrast to IVF, in ICSI the surrounding cumulus and corona cells must be removed completely, as only denuded oocytes can be successfully manipulated by the holding pipette. This ancillary effect of ICSI allows us to focus on the morphology of preimplantation development from the earliest stages. Early prognosis regarding the developmental fate of oocytes would help to limit a negative impact of culture conditions. However, little evidence is available that non-invasive selection at the oocyte stage (first polar body, granular cytoplasm) may be of prognostic value. Recently, certain patterns of pronuclei (number and the distribution of nucleoli) at the zygote stage were found to correlate with treatment outcome in IVF and ICSI cycles, offering an additional prognostic tool prior to cleavage. As there is evidence that embryo selection on day 2 or 3 based on morphological criteria (fragmentation, number of blastomeres, multinucleation, uneven cleavage) may be imprecise, patients might benefit from extended embryo culture to day 5. However, not all major chromosomal aberrations are incompatible with blastocyst formation, and prolonged culture *in vitro* does not exclusively select embryos with a normal chromosomal complement. Consequently, special care should be taken to minimize the presence of aneuploid concepti in culture. In addition, multiple selection at different stages of development will be required to filter out the correct 'candidate' embryo which will result in a healthy newborn.

Key words: aneuploidy/fragmentation/non-invasive selection/oocyte quality/polarity

Introduction

The success of assisted reproductive technologies (ART) depends on maximizing the efficiency of each individual step in the procedure. Improvements in controlled ovarian hyperstimulation, oocyte retrieval and culture conditions ensure high rates of fertilization and the availability of good-quality embryos for transfer. It is generally assumed that a certain role is attributed to the embryo transfer technique itself, but once an embryo resides within the uterus after transfer, implantation is governed by the quality of the endometrium and the conceptus (Paulson *et al*., 1990).

In-vitro fertilization

Historically, the evaluation of oocyte maturity in conventional IVF has been based on the expansion and radiance of the cumulus–corona complex surrounding the collected oocyte (Veeck, 1990). Using this approach, embryologists may quickly categorize oocytes as pre-ovulatory (metaphase II) when showing an expanded cumulus matrix and a 'sun-burst' corona radiata. A less expanded cumulus–corona complex may be designated as being of intermediate maturity, whereas the absence of an expanded cumulus might indicate oocyte immaturity (prophase I).

While this assessment provides a relatively close approximation of oocyte maturity, the method is unreliable in case of a disparity of maturation processes in oocyte and cumulus; for example, in patients revealing a plateau or drop in estradiol during controlled ovarian hyperstimulation (Laufer *et al*., 1984; Scott and Smith, 1998). The disparity in maturation is related to the use of exogenous hCG to induce maturation, and may result in incorrect timing of insemination in conventional IVF. In addition, no information regarding gamete quality on the day of follicle aspiration can be obtained because cumulus cells impair precise evaluation in IVF oocytes.

Intracytoplasmic sperm injection

With the introduction and establishment of ICSI in ART, this situation has changed entirely (Palermo *et al*., 1992; Van Steirteghem *et al*., 1993). In preparation for subsequent injection, the surrounding cumulus and corona cells must be

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removed, as only denuded oocytes can be successfully manipulated by the holding pipette. Denudation is carried out in standardized manner combining an enzymatic and a mechanical procedure. This ancillary effect of ICSI allows us to focus on the morphology of preimplantation development from the earliest stages.

On the other hand, ICSI is a rather invasive method that requires certain skills of the person performing it. In addition, the injection procedure itself could, in theory, damage cytoplasmic structures in the oocyte and result in impaired embryo development (Dumoulin *et al*., 2001). It has been speculated that the metaphase II (MII) spindle may be harmed irreversibly by either the injection pipette itself or by substances (e.g. polyvinylpyrolidone; PVP) gaining access during ICSI (Tsai *et al*., 2000; Barak *et al*., 2001). This would especially hold for oocytes in which the MII spindle cannot be predicted by the location of the first polar body (Hardarson *et al*., 2000). This problem may be elegantly overcome by employing recent advances in microscopical equipment (Polscope) which facilitate spindle imaging in mammalian oocytes without any detrimental effect to the gamete (Liu *et al*., 2000b; Wang *et al*., 2001).

Aspirating cytoplasm additionally increases the chance of disruption of the MII spindle. A defect in the spindle can result either in non-disjunction during second meiosis (Dumoulin *et al*., 2001) or irregular chromosome segregation (Rosenbusch and Sterzik, 1996). In addition, the aspiration of larger volumes of cytoplasm could involve the risk of accidental dislocation of portions of proteins (Antczak and Van Blerkom, 1997) or mitochondria (Edwards and Beard, 1997; Van Blerkom *et al*., 2000). Detrimental effects on both chromosomal structures and oocyte polarity could, theoretically, persist into later developmental stages.

Non-invasive selection

IVF and ICSI cycles have shown that, from her total pool of aspirated oocytes, a woman has only a limited number of gametes that are capable of generating a term pregnancy. This demonstrates the need for simple methods of preimplantation embryo assessment in the prediction of pregnancy rates. Even when relying on a well-established cryopreservation programme which, theoretically, would allow similar pregnancy rates combining fresh and frozen cycles, embryologists would benefit from adequate selection by avoiding the risk of cryoinjuries.

Several biochemical methods to assess human gamete and embryo quality have been described by analysing either the follicular fluid (Mendoza *et al*., 2002) or the metabolic activity of the embryo. The latter includes the measurement of pyruvate and glucose uptake (Leese *et al*., 1986; Gardner *et al*., 2001) and oxygen consumption (Magnusson *et al*., 1986). In addition, embryo viability may be prognostically predicted by measuring its platelet-activating factor production (Roudebush *et al*., 2002) or amino acid turnover (Houghton *et al*., 2002). These procedures are all complex and time-consuming however, and this severely limits their applicability in IVF laboratories.

Because of these potential difficulties, embryologists would much rather attend to the morphological appearance of gametes and embryos in order to obtain adequate information with regard to subsequent implantation behaviour. Consequently, the occurrence of high-order pregnancies, which is one of the major issues in ART—and one which causes the patient to face the dilemma of selective embryo reduction and an increased risk for both the mother and fetuses (Berkowitz *et al*., 1996; Roest *et al*., 1997)—may be avoided by selectively transferring a significantly smaller number of embryos.

In their search for such preimplantation embryos with optimal prognosis, embryologists may select at different stages of development. Selection of the female gamete can be applied in ICSI oocytes only, whereas selection can be carried out on both IVF and ICSI zygotes/embryos as soon as fertilization has been observed. It must be borne in mind that at the 4- to 8-cell stage it is largely a maternal selection with a possible paternal influence (sperm DNA integrity, aster formation), as in humans transcription from the embryonic genome starts at about day 3 (Braude *et al*., 1988; Taylor *et al*., 1997).

Oocyte quality (day 0)

One approach is to look for morphological aspects of the oocyte prior to ICSI and correlate them with the outcome of the treatment cycle. Oocytes retrieved from patients following controlled ovarian hyperstimulation show varying stages of meiotic maturity. Only oocytes at MII (extrusion of the first polar body) are suitable for ICSI, whereas oocytes at metaphase I (MI; first polar body not extruded) may only be used if few MII oocytes are available as their fertilization rate was found to be rather low (De Vos *et al*., 1999). Oocytes at prophase I (showing a germinal vesicle) cannot be used due to the diploid chromosomal set. In addition, giant MII-oocytes should be discarded as they will mostly result in digynic triploidy (Balakier *et al*., 2002; Rosenbusch *et al*., 2002).

Oocyte maturation from prophase I to MII starts with germinal vesicle breakdown when the flow of meiosis-arresting substances from the granulosa cells through the gap junctions into the oocyte is markedly reduced (Eppig, 1990). At this time, the oocyte must be fully grown and both nuclear and cytoplasmic maturation should be completed in a coordinated mode to ensure optimal conditions for subsequent fertilization. Disturbances or asynchrony of these processes may result in different morphological abnormalities, depending on whether nuclear (Eichenlaub-Ritter *et al*., 1995; Ebner *et al*., 2000) or cytoplasmic maturation (Hassan-Ali *et al*., 1998; Loutradis *et al*., 1999; Kahraman *et al*., 2000) has been affected.

Such disturbances may be caused by a reduced blood supply of the follicle during controlled ovarian hyperstimulation resulting in oxygen deficiency. Oocytes growing in such an environment were found to be of reduced viability (Chui *et al*., 1997). It has been shown that there is an association between a decrease in follicular blood flow and oocyte spindle as well as chromosomal defects (Van Blerkom *et al*., 1997). Hypoxia may also affect intracytoplasmic ATP content and cytoplasmic organization (Van Blerkom and Henry, 1992; Van Blerkom *et al*., 1995).

In such a scenario, with an inherent cytoplasmic defect or disorganization, the formation of polar axes which are associated

with morphogenesis throughout pre- and post-implantation stages (Edwards, 2000) may be impaired. Though not being visible at the time of ICSI, the possible effect of impaired polarity on subsequent development may be considerable. Unequal distribution of proteins (e.g. leptin and STAT3) originates from the specific positions of the planes of cell divisions that pass through such polarized protein domains (Antczak and Van Blerkom, 1997). Hence, hypothetically, impairment of polarity may be associated with atypical meridional or equatorial cleavage planes which will influence the corresponding pattern of inheritance of leptin/STAT3 as suggested previously (Antczak and Van Blerkom, 1997).

In addition, disproportionate segregation of mitochondria at the first cleavage division can result in human blastomeres in which the inherited complement of these cell organelles may be unable to contribute ATP at concentrations sufficient to maintain normal cell function (Van Blerkom *et al*., 2000). Suboptimal allocation of both proteins and mitochondria may cause cleavage arrest or a limited implantation potential.

It has been recognized that MII oocytes of good morphology should have a clear, moderately granulate cytoplasm, a small perivitelline space, an intact first polar body, and a colourless zona pellucida (De Sutter *et al*., 1996; Xia, 1997; Ebner *et al*., 2000). However, more than half of all oocytes collected show at least one morphological abnormality. These can be subdivided into cytoplasmic and extracytoplasmic abnormalities (Ebner *et al*., 2001b). The former include granularity or discoloration of the cytoplasm, aggregation of the smooth endoplasmic reticulum, vacuolization, and incorporations such as refractile bodies. To a certain degree, specific dysmorphic phenotypes in oocytes are likely to be a normal occurrence (Meriano *et al*., 2001), some of them having been suggested to reflect intrinsic defects that may adversely affect oocyte competence (Van Blerkom and Henry, 1992).

While one group (De Sutter *et al*., 1996) were not able to correlate oocyte morphology with fertilization rate or embryo quality, there is some evidence that outcome of ICSI may be severely affected by cytoplasmic appearance. Another group (Serhal *et al*., 1997) reported a pregnancy rate of 24% in patients with transfers derived solely from normal oocytes compared with those from oocytes with cytoplasmic abnormalities (3%), and similar results were reported elsewhere (Loutradis *et al*., 1999). In detail, a 29.4% pregnancy rate was achieved in transfers derived from normal oocytes, whereas only 5.5% of the pregnancies were achieved in the presence of oocytes showing dark cytoplasm. This finding was also supported by poor ongoing pregnancy rates (12.8%) in patients with granular cytoplasm of the oocytes (Kahraman *et al*., 2000). A significant decrease in preclinical pregnancy loss has been found (Alikani *et al*., 1995) in patients in whom none of the replaced embryos was dysmorphic in origin (20.0 versus 58.3% in oocytes with multiple dysmorphism). This negative impact on treatment outcome may be explained by a higher rate of aneuploidy found in dysmorphic oocytes (Plachot *et al*., 1988; Van Blerkom and Henry, 1992; Kahraman *et al*., 2000).

Different stimulation protocols and evaluation criteria may have contributed to the divergence in literature reports regarding the extent to which oocyte morphology, at light microscopy level, correlates with ICSI outcome (Balaban *et al*., 1998).

Extracytoplasmic abnormalities include irregularities in oocyte shape, enlargement of the perivitelline space, presence of debris within this space, fragmentation of the first polar body, and abnormal consistency of the oolemma and the zona pellucida. Some of these features are associated with a decreased survival rate of oocytes after ICSI (Ebner *et al*., 2001b), but not with fertilization and embryo quality.

However, an intact first polar body showing a smooth surface was found to be of positive prognostic value in terms of fertilization and embryo quality (Ebner *et al*., 2000; 2002), as well as implantation and pregnancy rate (Ebner *et al*., 1999; 2002). This supports the findings of others (Xia, 1997) who demonstrated a negative effect of oocytes with fragmented first polar bodies, a large perivitelline space and cytoplasmic inclusions on the percentage of zygotes and embryo quality. Prognostic relevance of first polar body morphology has also been suggested by data for oocytes matured *in vitro* (Mikkelsen and Lindenberg, 2001).

Extrusion of the first polar body indicating the end of meiotic maturation can easily be examined. It is thought that ageing *in vivo* in MII before aspiration may lead to degeneration of the first polar body, and that the resultant overmaturity of such oocytes may further contribute to a diminished developmental potential (Eichenlaub-Ritter *et al*., 1995; Ebner *et al*., 1999; 2000).

Zygote quality (day 1)

Time-lapse video cinematography has shown that normal fertilization follows a definite course of events, though timing of these events may vary between oocytes (Payne *et al*., 1997). In the aforementioned study, approximately 90% of the oocytes showed circular waves of granulation within the ooplasm after ICSI (periodicity of 20–53 min). During this granulation phase the sperm head decondensed. Subsequently, the second polar body was extruded, which was followed by the central formation of the male pronucleus. At about the same time, the female pronucleus formed and was drawn towards the male pronucleus until the two abutted. Both pronuclei then increased in size, and their nucleoli moved around and arranged themselves near the common junction.

Within the nucleus, nucleoli form in so-called ‘nucleolus-organizing regions’ located on the chromosomes of the oocyte (King *et al*., 1988). The nucleoli are the active sites of rRNA synthesis. During the course of development, nucleoli tend to fuse due to an increase in protein synthesis (Tesarik and Kopecny, 1989; Payne *et al*., 1997). Asynchrony in the formation and polarization of nucleoli may severely impair further development of the preimplantation embryo (Van Blerkom, 1990; Tesarik and Greco, 1999; Scott *et al*., 2000). Consequently, good-quality embryos can arise from oocytes that had more uniform timing from injection to pronuclear abuttal (Payne *et al*., 1997).

It must be borne in mind that in about 1% of all zygotes with two polar bodies, pronuclei are not visible at the time of morphological assessment (Manor *et al*., 1996). This may be due to either an abnormal developmental speed or to intense granulation eventually hiding pronuclei. Normal chromosomal status was observed in 57% of such undocumented zygotes (Munné and Cohen, 1998).

Another irregularity in pronuclear formation is the presence of two unevenly sized pronuclei. The average difference in pronuclear size was approximately 10 μm (Sadowy *et al*., 1998). This dysmorphism has been documented in less than 2% of all oocytes,

Table I. Summary of treatment outcome (clinical pregnancies) in relation to zygote scoring according to Tesarik and Greco (1999)

Reference	Method	Day	Transfer All pattern 0	At least 1 pattern 0	No pattern 0
Tesarik *et al.* (2000)	ICSI	2	44/98 (44.9)[a]	47/199 (23.6)	17/77 (22.1)[a]
Wittemer *et al.* (2000)	IVF/ICSI	3	46/109 (42.2)[b, c]	31/87 (35.6)[b]	13/66 (19.7)[c]
Balaban *et al.* (2001c)	ICSI	5	13/15 (86.6)[d, e]	29/55 (52.7)[e, f]	3/16 (18.7)[d, f]
Montag and Van der Ven (2001)	IVF/ICSI	2–5	ND	311/866 (35.9)[g]	426/1639 (26.0)[g]
Salumets *et al.* (2001)	IVF/ICSI	2/3	16/56 (28.6)	ND	23/88 (26.1)

Values in parentheses are percentages.
[a, e, f], $P < 0.05$.
[b, c, d, g], $P < 0.01$.
Day = day of transfer; ND = no data available.

but in 14% of all patients. There was a close relationship to mosaicism since only 13% were found to be euploid (Sadowy *et al*., 1998).

Pronuclear grading

Following a retrospective analysis of 92 pronuclear embryo transfers, a zygote score was developed for IVF laboratories which considered the alignment of nucleoli at the junction of the two pronuclei as a selection criterion for embryo transfer (Scott and Smith, 1998). In addition, the score included the appearance of cytoplasm and timing of nuclear membrane breakdown, for which prognosis was most positive if the cytoplasm showed a clear halo and was heterogeneous in appearance and if the zygote progressed at least to pronuclear membrane breakdown within 24–26 h after insemination.

However, others (Tesarik and Greco, 1999) claimed to predict preimplantation development by focusing exclusively on the number and the distribution of nucleoli (nucleolar precursor bodies; NPB) in each pronucleus. These authors considered interpronuclear synchrony, evaluated at 12–20 h after IVF/ICSI, to be more important than the actual NPB polarity at the site of pronuclear apposition, ss they presumed that polarization of nucleoli was not evident from the beginning of pronuclei formation but appeared progressively with time (Tesarik and Kopecny, 1989). According to these authors (Tesarik and Greco, 1999) the optimal synchronized pattern yielded 37.3% of good-quality embryos compared with only 27.8% of all other patterns.

This contrasted, at least in part, to the prospective results of another group (Montag and Van der Ven, 2001) who introduced an additional subclassification of the pronucleus patterns with the best prognosis (Tesarik and Greco, 1999) to include one group showing NPB polarization at the area of pronuclear contact, and one without. The former was found to be the subgroup of zygotes with the best prognosis, since a significantly increased implantation rate could be seen (20.5%) if at least one embryo was derived from this optimal pronuclear pattern (16–20 h after insemination) compared with transfers involving no such patterns (15.7%).

Based on the results of these retrospective studies (Scott and Smith, 1998; Tesarik and Greco, 1999), several other groups have investigated this subject (Ludwig *et al*., 2000; Tesarik *et al*., 2000; Wittemer *et al*., 2000; Montag and Van der Ven, 2001). Although the grading systems differed slightly between these reports, the conclusion was common in that zygotes showing pronuclei with approximately the same number and alignment of nucleolar precursor bodies had a good prognosis in terms of subsequent implantation (Table I). An increased incidence of subsequent blastocyst formation in zygotes with an optimal pattern of the pronuclei (Scott *et al*., 2000; Balaban *et al*., 2001c) seemed to be consistent with the reported increase in terms of pregnancy rate.

One study (Salumets *et al*., 2001) failed to show any correlation between zygote score and pregnancy rate, however. This was of particular interest because the analysis included only single embryo transfers and, consequently, the actual implantation potential could be estimated. Although two different scores were applied (Scott and Smith, 1998; Tesarik and Greco, 1999), no correlation with treatment outcome could be demonstrated. This discrepancy in the literature results may be explained by the use of different culture media, stimulation protocols and differences in timing of fertilization assessments, for example the inclusion of early cleavage in the scoring system used (Scott and Smith, 1998).

Interestingly, a significant difference between IVF and ICSI cycles with regard to the number of good pattern (pattern 0; according to Tesarik and Greco, 1999) was reported (Montag and Van der Ven, 2001). In detail, superior pronuclear patterns could be observed in ICSI cycles, but this phenomenon may have been due to an accelerated course of development in ICSI (Nagy *et al*., 1998; Sakkas *et al*., 1998). Zygotes showing this most advanced stage of nuclear polarization seem to reach that stage earlier after ICSI than after conventional IVF (Montag and Van der Ven, 2001). However, the study did not evaluate the position of the pronuclei relative to the presumed polar axis. This arrangement has been reported to relate to embryo quality (Garello *et al*., 1999). It has been suggested (Edwards and Beard, 1997) that the oocyte may establish this polarity by either ooplasmic or pronuclear rotation towards the second polar body. Such a re-setting of a new axis after fertilization is governed by cytoplasmic contraction waves organized by the sperm centrosome (Edwards and Beard, 1997). Embryos

unable to achieve optimal pronuclear orientation, possibly due to shorter cytoplasmic waves (Payne *et al*., 1997), may exhibit poor morphology, for example uneven cleavage or fragmentation (Garello *et al*., 1999).

Cytoplasmic appearance

Immediately prior to pronuclear growth, the organelles contract from the cortex towards the centre of the oocyte, leaving a clear halo (Payne *et al*., 1997). This phenomenon, which is the manifestation of a microtubule-mediated withdrawal of mitochondria and other cytoplasmic components such as the endoplasmic reticulum and Golgi apparatus, has been documented in humans and other mammals (Bavister and Squirrell, 2000).

The presence of a cytoplasmic halo effect may be recognized in two-thirds of all oocytes (Salumets *et al*., 2001; Stalf *et al*., 2002). Although the physiological relevance of this translocation of cell organelles requires further elucidation, the presence of a halo was found to correlate significantly with embryo quality but not with outcome (Salumets *et al*., 2001). In contrast, a significantly higher pregnancy rate (44.0%) was observed in halo-positive women compared with those who were halo-negative (28.3%) (Stalf *et al*., 2002). This apparently contradictory result may be explained by the fact that no subdivision of halo effects was used; indeed, it has been suggested recently that an extreme halo might have a negative effect on blastocyst development (Zollner *et al*., 2002).

Early cleavage

Early cleavage at 25–27 h after insemination was reported to be very effective in aiding the selection of viable embryos for transfer (Fenwick *et al*., 2002). One group (Shoukir *et al*., 1997) observed early cleavage in 18.9% of all IVF cycles having a pregnancy rate (33.3%) which was double that of cycles without early-cleaving zygotes (14.7%). Similar results were achieved in ICSI patients (Sakkas *et al*., 1998), where 61.4% of the cycles had zygotes that cleaved within 27 h, leading in turn to a pregnancy rate of 25.9% compared with a rate of 3.2% in patients without early cleavage. The same authors stated that early cleavage is more likely to be intrinsic to the oocyte of a particular patient than to result from the different timing of fertilization in IVF and ICSI (Sakkas *et al*., 2001). However, it must be considered that early cleavage is related to female age and can be increased in ICSI zygotes compared with those obtained by IVF (Lundin *et al*., 2001). This is because the direct injection of a spermatozoon bypasses most of the fertilization steps (including the acrosome reaction and binding to the zona pellucida) and results in a shorter fertilization time (Nagy *et al*., 1998). Hence, it may be better either to inseminate oocytes earlier or screen IVF zygotes later in order to obtain comparable data (Lundin *et al*., 2001).

This critical time for selection can be extended to up to 29 h after insemination (Bos-Mikich *et al*., 2001). One advantage of embryo selection based on the timing of first cleavage is that this is a clearly visible event, whereas pronuclear morphology may vary during the dynamic process of syngamy (Tesarik and Kopecny, 1989). The reason why early cleavage yields better quality embryos and higher pregnancy rates (Lundin *et al*., 2001) is unknown, but it may be speculated that such zygotes derive from oocytes with adequately synchronized cytoplasmic and nuclear maturation.

Early cleavage may also indicate those zygotes with euploid sets of chromosomes (Hardarson *et al*., 2001), since a recent study (Lundin *et al*., 2001) showed that early cleavage rate in 3 pronuclear (3PN) zygotes was only 12% compared with 26.9% in 2PN zygotes.

Embryo quality (day 2 or 3)

During the early years of conventional IVF, the impossibility of evaluating oocyte quality, coupled with a lack of prognostic criteria at the zygote stage, led to the introduction of embryo grading systems being used on day 2. These systems are based mainly on the number and shape of the blastomeres, as well as on the percentage of fragmentation (Veeck, 1990).

A cumulative embryo scoring system (CES) was proposed (Steer *et al*., 1992) which enabled IVF units both to select the optimal number of embryos for transfer and to reduce the incidence of multiple pregnancies. The CES combines embryo morphology, cleavage rate and number of embryos transferred into a single figure, representing their collective quality, and can be applied to embryo selection before transfer. When others (Visser and Fourie, 1993) substantiated the applicability of the CES in an IVF programme, pregnancy rates were found to increase from 4% (with scores between 1 and 10) to 35% in the 41- to 50-year age group. A score of 20 was the criterion for subdividing patients into poor and good pregnancy prognosis groups.

Numerous studies have indicated an influence of the number and quality of embryos transferred to the uterus on implantation rate, pregnancy rate and multiple pregnancy rate. However, a major problem confounding the results of all these studies was the impossibility of knowing which embryo actually implanted, as the embryo transfers were not homogeneous with regard to embryo morphology, and this may have led to contradictory results (Hoover *et al*., 1995).

Fragmentation

Recently, data from single (Giorgetti *et al*., 1995) and homogeneous double and triple embryo transfers (Ziebe *et al*., 1997) confirmed that on day 2 after transfer, 4-cell embryos—even when showing minor fragmentation—should be preferred to good-quality 2-cell embryos.

This is in accordance with previously published findings (Van Royen *et al*., 1999) which summarized the characteristics of a top quality embryo as: an absence of multinucleated blastomeres; four or five blastomeres on day 2; seven or more cells on the following day; and <20% fragments.

It is speculated that in the case of moderate fragmentation, different temporal or spatial patterns of fragmentation have a more profound effect on the developmental fate of the embryo than the occurrence of fragments *per se* (Alikani *et al*., 1999; Van Blerkom *et al*., 2001). On the other hand, certain phenotypes of minor fragmentation may disappear during in-

vitro culture, either by lysis or by resorption (Hardarson *et al*., 2001; Van Blerkom *et al*., 2001), thereby providing for a high-quality, stage-appropriate embryo on the day of transfer.

This disappearance of fragments may be imitated by microsurgical removal of the vast majority of anuclear fragments. In such cases, improved outcome is attributed to the restoration of the spatial relationship between the blastomeres within an embryo (Alikani *et al*., 1999). It is indeed conceivable that fragments situated near an assumed cleavage axis could exert a detrimental impact on polarity of the cleaved embryo, thereby limiting its chance for subsequent implantation. Assuming that polarized molecules have been selectively removed through fragmentation (Almeida and Bolton, 1996; Antczak and Van Blerkom, 1999), polarity would be impaired irreversibly.

Few reports exist of pregnancies derived from heavily fragmented embryos (Scott *et al*., 1991; Staessen *et al*., 1992; Alikani *et al*., 1999; Ebner *et al*., 2001a), and there is some evidence that perinatal outcome of such pregnancies from poor-quality embryos (⩾50% fragmentation) is worse when compared with pregnancies derived from embryos with fewer anuclear fragments (Ebner *et al*., 2001a).

Heavily fragmented embryos are known to present a higher rate of chromosome abnormalities, in particular mosaicism (Plachot *et al*., 1987; Pellestor *et al*., 1994; Munné and Cohen, 1998). It is assumed that, to a certain degree, affected blastomeres can be entirely excluded from further development by selective fragmentation (Warner *et al*., 1998). Cellular fragmentation is likely to be related to programmed cell death (Jurisicova *et al*., 1996; Warner *et al*., 1998). Two apoptosis-related gene families could be detected in human embryos, namely bcl-2 and bax (Warner *et al*., 1998; Liu *et al*., 2000a), the ratio of which is expected to determine whether a blastomere survives or is eliminated. Others (Antczak and Van Blerkom, 1999) questioned a direct association between fragmentation and apoptosis, but speculated that fragments *per se* may provide a trigger for apoptosis if a certain level of developmentally important proteins is eliminated from their polarized domains.

Uneven cleavage

One generally underestimated morphological feature of a cleaved embryo is uneven cellular cleavage. Unevenly sized blastomeres may be the manifestation of an uneven distribution of genetic material. Actually, a significantly higher number of blastomeres affected by numeric chromosomal aberrations has been reported in unevenly cleaved embryos (29.4%) as compared with evenly cleaved ones (8.5%) (Hardarson *et al*., 2001). As a consequence, unevenly cleaved embryos have a reduced potential to implant (Giorgetti *et al*., 1995; Ziebe *et al*., 1997; Hardarson *et al*., 2001).

In addition, uneven blastomere size within an embryo could be correlated with an increased rate of multinucleated blastomeres (Hardarson *et al*., 2001). Multinucleation may result from a failure in cytokinesis during early cleavage (Hardy *et al*., 1993) and is expected to result in concepti with developmental incompetence (Kligman *et al*., 1996), a situation which is also supported by the low implantation potential of such embryos (Pelinck *et al*., 1998).

Blastocyst stage (day 5 or 6)

There is evidence however that embryo selection on day 2 or 3 based on morphological criteria may be imprecise, resulting in the transfer of embryos that are abnormal or arrest at later developmental stages (Rijnders and Jansen, 1998; Graham *et al*., 2000; Milki *et al*., 2002). Consequently, some patients might benefit from extended culture to day 4 (Tao *et al*., 2002) or day 5.

It must be borne in mind that any prolongation of in-vitro culture includes the risk of altering culture conditions. Due to repeated morphological assessment of the oocytes and embryos, changes in temperature, additional light exposure and pH-shifts may occur which might lead to a further deviation from in-vivo conditions. In addition, the quality of the preimplantation embryo may be affected by prolonged culture, for example alterations to the zona pellucida (De Vos and Van Steirteghem, 1999).

It has been shown over the past decades that human embryos can be cultured to day 5 in a variety of culture conditions. However, these blastocysts either did not fully expand (Edwards and Beard, 1999) or failed to implant in adequate number (Gardner and Lane, 1997). A more successful attempt to produce more viable blastocysts *in vitro* was by co-culture (Ménézo *et al*., 1990); nevertheless, rates of implantation and pregnancy were still not comparable with those achieved with blastocysts developed *in vivo*, which were flushed from the uterus and retransferred (Buster *et al*., 1985).

The recent improved understanding of both the different metabolic needs of the cleavage- and blastocyst-stage embryos and the physiological changes in the female reproductive tract (Gardner *et al*., 1996) has resulted in the development of a new generation of sequential embryo culture media (Gardner and Lane, 1998; Ménézo *et al*., 1998). It is important to note that this approach will not necessarily yield higher numbers of blastocysts but, more importantly, blastocysts that are more viable as assessed by increased implantation rates.

Improved embryo selection

One of the potential advantages of prolonged culture *in vitro* is the non-invasive selection of embryos with a presumed increased implantation potential.

Prior to day 3, embryo development is primarily governed by maternal transcripts; consequently, when embryos are selected at the 4- to 8-cell stage, the embryonic genome has only just begun to be transcribed (Braude *et al*., 1988; Taylor *et al*., 1997). Only by culturing embryos past embryonic genome activation and up to blastocyst stage is it possible to identify those embryos which have had a developmental block on day 2 or 3. Therefore, the most competent embryos reaching the blastocyst stage may be selected for transfer.

It is important to note that embryo development on day 5 or 6 may vary from a retarded stage of compaction to the blastocyst stage. The quality of the latter is determined by the formation of both a distinct blastocoele and a trophectoderm (TE) consisting of sickle-shaped cells. In addition, size of the inner

Table II. Comparison of day 3 and day 5 transfer for treatment outcome (implantation/clinical pregnancies). All studies evaluated both IVF and ICSI cycles

Reference	Study design	Patient prognosis	Day 3			Day 5		
			n	IR	PR	*n*	IR	PR
Gardner *et al.* (1998)	Prospective	Good	3.7[a]	30.1[b]	31/47 (66.0)	2.2[a]	50.5[b]	32/45 (71.1)
Cruz *et al.* (1999)	Retrospective	Bad	5.4[c]	3.4[d]	2/22 (9.1)[e]	3.1[c]	11.3[d]	6/15 (40)[e]
Marek *et al.* (1999)	Retrospective	Unselected	3.0[f]	23.3[g]	171/463 (36.9)[h]	2.5[f]	32.4[g]	137/292 (46.9)[h]
Coskun *et al.* (2000)	Prospective	Unselected	2.3	21.3	39/101 (38.6)	2.2	23.9	39/100 (39.0)
Huisman *et al.* (2000)	Prospective	Unselected	1.9	14.4	149/590 (25.3)	1.9	15.5	197/709 (27.8)
Balaban *et al.* (2001a)	Prospective	Bad	5.2[i]	5.9[j]	44/162 (27.2)	2.4[i]	15.0[j]	53/158 (33.5)
Wilson *et al.* (2002)	Retrospective	Unselected	2.7[k]	27.0[l]	172/358 (48.1)[m]	2.0[k]	43.0[l]	284/494 (57.5)[m]
Utsunomiya *et al.* (2002)	Prospective	Unselected	2.9	11.7	40/152 (26.3)	3.0	9.2	30/121 (24.8)

Values in parentheses are percentages.
[a], [b], [f], [g], [h], [l], [m], $P < 0.01$.
[c], [d], [e], $P < 0.05$.
[i], [j], [k], $P < 0.001$.
IR = implantation rate; *n* = mean number of embryos transferred; PR = pregnancy rate.

cell mass (ICM) was found to be most important with regard to successful implantation (Gardner and Schoolcraft, 1999; Balaban *et al*., 2000; Gardner *et al*., 2000a; Richter *et al*., 2001). In detail, the ICM was scored according to the cell number (Gardner *et al*., 2000a). A tightly packed ICM with many cells represents the best grade (A), whereas grade B shows a loosely grouped cell mass with several cells. The remaining ICM with very few cells was the worst grade, C. In parallel, trophectoderm was considered of good quality if many cells formed a cohesive epithelium. By using this scoring system to select for transfer excellent implantation rates could be achieved, for example 50% in single blastocyst transfer.

This is, at least in part, in line with the work of others (Richter *et al*., 2001) who applied quantitative measurement to expanded blastocysts. Blastocyts with a slightly oval ICM and a size of >4500 μm^2 implanted at a higher rate compared with more round and smaller ICMs.

There is evidence that transfer at the blastocyst stage may increase rates of implantation and pregnancy (Gardner *et al*., 1998; Schoolcraft *et al*., 1999). However, three out of five prospective studies (see Table II) could not identify significantly higher rates of implantation and clinical pregnancy (Coskun *et al*., 2000; Huisman *et al*., 2000; Utsunomiya *et al*., 2002). These results do not support the effectiveness of prolonged culture to day 5.

Women having at least 10 follicles available on the day of oocyte retrieval may benefit most from blastocyst culture (Schoolcraft *et al*., 1999), but there is a tendency to apply day 5 transfer to non-selected patient groups (Marek *et al*., 1999; Wilson *et al*., 2002). Blastocyst culture and transfer may help to overcome implantation failure even in patients with poor prognosis—for example patients with several failed treatments (Cruz *et al*., 1999) or with exclusively bad quality embryos on day 2 or 3 (Balaban *et al*., 2001a). The results of the most relevant studies comparing day 3 and day 5 transfers are summarized in Table II.

However, one of the last unsolved problems in blastocyst culture is the patient cohort without adequate response to controlled ovarian hyperstimulation. It seems logical that the chance of achieving at least one blastocyst for transfer decreases with the number of available oocytes or zygotes. Therefore, there is a certain need for prognostic parameters on day 2 or 3 which would allow the formation of at least one blastocyst to be predicted. It has been reported that each blastocyst developing *in vitro* appears to have the same viability, regardless of whether it is the only blastocyst that developed or is one of ten such blastocysts (Gardner *et al*., 1998; Shapiro *et al*., 2002).

Predictors of blastocyst development and subsequent viability

The possibility of blastocyst transfer on day 5 depends primarily on the number of oocytes retrieved (Jones *et al*., 1998; Scholtes and Zeilmaker, 1998; Langley *et al*., 2001). In addition, the number of blastocysts available on day 5 showed a significant correlation to pregnancy rate (Gardner *et al*., 2000b). This, together with the finding that the blastocyst development of untransferred embryos is positively related to the outcome of the treatment (Fisch *et al*., 1999), derives from the possibility of selecting the highest scoring blastocysts for transfer.

One group (Racowsky *et al*., 2000) found the number of 8-cell embryos on day 3 to be a key determinant for selecting embryo or blastocyst transfer. It is important to note that it is the number of blastomeres (Shoukir *et al*., 1998a; Langley *et al*., 2001) that is reponsible for this correlation rather than the degree of fragmentation, because day 3 morphology seems not to be an exact predictor of blastocyst formation. It has also been reported (Rijnders and Jansen, 1998) that only 51% of all blastocysts transferred on day 5 had been pre-selected on day 3. This is in line with the findings of others (Graham *et al*., 2000; Milki *et al*., 2002), who selected 48% and 42% respectively of their pre-selected day 3 embryos for blastocyst transfer. Hence,

a distinct group of patients may benefit from prolonged culture to day 5.

Elimination of high-order multiple gestations

The ability to identify embryos having an increased potential for implantation on day 5 helps to limit the risk of multiple pregnancies without decreasing the pregnancy rate (Huisman *et al*., 2000). Triple pregnancies can be reduced by strict limitation of transfer to a maximum of two blastocysts (Milki *et al*., 1999). In addition, there is evidence that blastocyst culture may favour monozygotic twinning due to alterations of the zona pellucida and/or the hatching process (Behr *et al*., 2000; Da Costa *et al*., 2001; Tarlatzis *et al*., 2002).

Consequently, every effort should be made to switch to a single blastocyst transfer strategy (Schoolcraft *et al*., 1999; Gardner *et al*., 2000a). Transfer of one blastocyst only may be a feasible option taking into account the prognostic potential of blastocyst quality (Gardner and Schoolcraft, 1999; Balaban *et al*., 2000; Gardner *et al*., 2000a; Richter *et al*., 2001). Since more than one blastocyst will develop in many patients, there is a desperate need for an optimized blastocyst freezing protocol to increase the cumulative pregnancy rate.

Indirect selection of chromosomal abnormalities

There is increasing evidence that ICSI patients have a lower percentage of embryos that form blastocysts compared with couples undergoing conventional IVF (Janny and Ménézo, 1994; Shoukir *et al*., 1998b; Dumoulin *et al*., 2000). Manifestation of a negative paternal effect on preimplantation development caused by abnormal spermatozoa may either be associated with the role of the sperm in sperm aster formation (Asch *et al*., 1995), or occur at the genetic level during embryonic genome activation (Shoukir *et al*., 1998b). In addition, possible harm to further development may arise by using spermatozoa showing nuclear DNA damage (Sakkas *et al*., 2000; 2002). However, a recent study reported that blastocyst development *in vitro* rather reflects the competence of the oocyte cytoplasm and is relatively independent of a paternal genomic effect (Banerjee *et al*., 2000).

A higher rate of euploidy in early-cleaving embryos (Hardarson *et al*., 2001), as well as a presumed correlation between slow cleavage rates and chromosomal abnormality (Munné *et al*., 1995), are two arguments for the existence of a mechanism which selectively eliminates chromosomally affected embryos *in vitro*. However, a possible allocation of euploid cells to the ICM and aneuploid cells to the TE (Hardy *et al*., 1989) was doubted when the mosaicism rate in the ICM (10.5%) was shown to be similar to the overall mosaicism rate (Evsikov and Verlinsky, 1998).

According to these authors (Evsikov and Verlinsky, 1998), the degree of mosaicism up to the morula stage is increased compared with that found in the blastocyst. Clearly, a mechanism of self-destruction is triggered at the time when ICM and TE lineages diverge in order to form the highly polarized, bilaterally symmetric blastocyst (Magli *et al*., 2000).

Although aneuploid embryos are more likely to block in their development between activation of the embryonal genome and day 5 than those diagnosed as euploid (Magli *et al*., 2000), not all major chromosomal aberrations are incompatible with blastocyst formation, and prolonged culture *in vitro* does not exclusively select embryos with a normal chromosomal complement (Magli *et al*., 2000; Sandalinas *et al*., 2001). While the majority of chromosomally abnormal blastocysts will be lost either prior to or following implantation, some will develop to term. It was shown that a significantly lower number of embryos developed to morula or blastocyst stage in aneuploid embryos (30/73; 41.1%) compared with euploid embryos (45/70; 64.3%) (Magli *et al*., 2000). Similar results were reported by others (Sandalinas *et al*., 2001), with a 65% (120/184) rate of developmental block prior to the compaction stage in aneuploid embryos compared with 28% (9/32) in euploid embryos.

Taking into consideration that, after controlled ovarian hyperstimulation, approximately 50% of all mature oocytes (Van Blerkom and Henry, 1992) and embryos (Munné *et al*., 1995; Magli *et al*., 2000) are chromosomally impaired, the risk to select an aneuploid conceptus may at least be reduced if transfer is performed at the blastocyst stage, since it has been found that aneuploid embryos have a significantly reduced capacity to form blastocysts (Magli *et al*., 2000; Sandalinas *et al*., 2001).

Conclusion

In general, non-invasive selection methods based on the morphological assessment of concepti may be applied to day 2 or 3 embryos as well as to day 5 blastocysts. However, prolonged culture to day 5 offers an additional possibility to select which is highly informative, since the quality of the ICM and TE can be assessed exactly (et al., 2000a). Hence, embryologists can select—from several blastocysts with similar quality having formed on day 5—those which have shown beneficial morphological characteristics at the oocyte, zygote and/or cleavage stage.

When weighing up the pros and cons of prolonged in-vitro culture, it must be borne in mind that any prolongation of culture may increase the risk of introducing additional factors that might negatively influence preimplantation development. In addition, in most patients the cumulative pregnancy rate will be lower as blastocyst freezing is less successful than cryopreservation at earlier embryonic stages.

As not all prospective data support prolonged culture, the recommendation of blastocyst transfer should only be made in patients that have sufficient embryos with a good prognosis of blastulation. This decision should be made under the premise that there are no other factors that might potentially affect blastocyst formation, including the woman's age (Shapiro *et al*., 2002) or male subfertility (Janny and Ménézo, 1994; Jones *et al*., 1998; Shoukir *et al*., 1998b; Pantos *et al*., 1999; Balaban *et al*., 2001b).

Independently of the day of transfer, correct identification of viable concepti can be carried out by combining two different approaches.

Screening for aneuploidy

First, repeated screening for morphological features attributed to chromosomal aberrations should be carried out from the oocyte stage onwards because spontaneous post-zygotic errors may occur and persist *in vitro* throughout the preimplantation development (Bielanska *et al*., 2002).

As previously reported (Balakier *et al*., 2002; Rosenbusch *et al*., 2002), the exclusion of giant oocytes from ART trials is recommended in order to reduce the risk of digynic triploidy. Oocytes with a centrally located granular cytoplasm (Kahraman *et al*., 2000), as well as undocumented zygotes (Manor *et al*., 1996) showing two polar bodies but no pronuclei, should only be transferred if insufficient dipronucleated embryos are available. In addition, zygotes with uneven pronuclear size must be discarded as 87% were considered abnormal (Sadowy *et al*., 1998). Last—but not least—embryos with uneven blastomeres and/or multinucleated blastomeres at cleavage stages (day 2–3) should be eliminated in order to prevent accidental transfer in case of blastocyst formation (Hardarson *et al*., 2001). This 'negative' selection approach might help to reduce presence of chromosomally abnormal embryos or blastocysts in culture compared to an 'unselected' pool of concepti.

Positive predictors of preimplantation development

In addition, double or even triple selection at different stages of development will be required to determine whether a certain benefit attributed to earlier stages will persist into later stages. Such serial observations must be made using individual culture of embryos in order to provide for correct identification. If separate culture of embryos is taken as a requirement, then only a few studies cited in this review provide prognostically relevant evidence in terms of implantation behaviour. For the day of oocyte collection, this would include the finding that an intact first polar body is of positive prognostic value (Xia, 1997; Ebner *et al*., 2000). At the zygote stage, both the presence of a halo-like cytoplasmic appearance (Stalf *et al*., 2002; Zollner *et al*., 2002) and pronuclear morphology (Tesarik and Greco, 1999; Scott *et al*., 2000; Wittemer *et al*., 2000; Montag and Van der Ven, 2001) were shown worthy of inclusion as selection criteria. At the cleavage stage, the number of studies with single culture of embryos is limited, because either different embryo grades were cultured in groups or cumulative scores were applied. The same will hold for the blastocyst stage, since most published reports did not apply individual culture routinely. In fact, only two studies (Scott *et al*., 2000; Balaban *et al*., 2001c) have focused on the blastocyst development of morphologically different zygotes, thus indicating that different selection methods target the same embryo.

In conclusion, the decision of whether to transfer at cleavage stages or at the blastocyst stage is very complex and should be based on both the detailed evaluation at early embryonic stages as well as individual prognosis of blastocyst formation. The establishment of an adequate cryopreservation programme may lead to similar cumulative pregnancy rates in embryo transfers compared with blastocyst transfer.

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