Embryo culture, assessment, selection and transfer

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Embryo morphology and growth rate

Preparation for mitotic division begins with two haploid pronuclei duplicating their DNA, the two pronuclei then come together and syngamy occurs at 20–34 hours after insemination followed by division at approximately 35.6 hours which results in the formation of two diploid blastomeres containing approximately half the cytoplasm of the fertilized oocyte *(1)*. Mitotic division during the preimplantation period results in a rapid increase in blastomere numbers, as there is no cell growth phase prior to mitosis. This results in daughter cells that are smaller than parent blastomeres, leading to a progressive reduction in individual blastomere volume. With the use of co-culture methods and the advent of complex and sequential culture media, the embryo quality and growth rate have improved and there are variations in the cell number and morphology of the embryo depending on the culture media and culture conditions employed.

Once the sperm has fertilized the oocyte, the centriole and microtubules arising from the sperm bring the male and female pronuclei into juxtaposition *(2)*. Pronuclear alignment occurs 16–18 hours post-insemination and failure to do so is indicative of one or more fertilization events failing to occur *(3)*. Nucleoli are visible within the respective pronuclei during this period and are responsible for rRNA synthesis, which resumes at fertilization *(4,5)*. The nucleoli or nuclear precursor bodies show various patterns of presentation which are suggestive of the chromosomal status and developmental inferiority of the embryo. Tesarik and Greco *(6)* examined the pronuclear morphogenesis in an attempt to determine embryo viability dependent on distribution and alignment of the nucleolar precursor bodies in the respective nuclei. Embryos resulting from certain distribution patterns exhibited greater viability and lower rates of embryonic arrest. This group constituted the "normal" pronuclear stage morphology, with the remaining groups described as abnormal, attributed to asynchronous pronuclear development, which the authors believe is harmful to embryo viability. Pronuclear alignment in relation to the polar body axis, positioning of the nucleoli, and cytoplasmic consistency have also been shown to affect embryo viability. Sadowy *et al.* *(7)* suggested that size variation between the two pronuclei may indicate chromosomal anomalies. The authors showed an increase in embryonic arrest in zygotes with pronuclei that differ by four microns from each other. A higher incidence of multinucleation and mosaicism was also observed in zygotes with dysmorphic pronuclei. Recent evidence suggests that polarity in mammalian oocytes, including human oocytes, exists and can be evidenced in the distribution of surface structures and molecules, cytoplasmic organelles, and RNA. The

animal pole of the oocyte may be estimated by the location of the first polar body, whereas after fertilization, the second polar body marks the so-called embryonic pole *(8)*. In human oocytes, there is a differential distribution of the molecules leptin and STAT3 to the cortical region of the oocytes containing the animal pole and subsequent mitotic divisions produce blastomeres with different allocations of these polarized molecules *(9)*. Prior to syngamy, pronuclei can be observed to rotate within the ooplasm, directing their axes towards the second polar body *(10)*. Theoretically, embryos that do not achieve an optimal pronuclear orientation may exhibit cleavage anomalies that may be observed as poor morphology, uneven cleavage or fragmentation. Garello *et al. (11)* demonstrated that there is no relationship between the magnitude of the angle between the first and second polar body and subsequent embryo morphology, probably due to the fact that the first polar body is capable of moving over an angle of 30° while the second polar body remains stationary. However, these authors provided some evidence in support of the hypothesis that the orientation of the pronuclei relative to the polar bodies relates to the subsequent morphological grade of the embryo *(11)*. Although the absolute magnitude of the angle between the pronuclei and polar bodies did not relate to embryo grade, the angle increased significantly with decreasing embryo quality.

Tripronuclear oocytes are usually formed by dispermic fertilization and, as a result of the formation of a tripolar spindle, the majority (62%) divide into 3 cells with severely abnormal chromosome numbers *(12)*. Some oocytes with a bipolar spindle (24%) form triploid embryos and some (14%) regain a diploid karyotype by expulsion of a set of chromosomes in a nucleated fragment *(12)*. As a consequence, it is recommended that tripronuclear oocytes are discarded.

Monopronuclear oocytes may be diploid and some will develop to a blastocyst and to term when transferred to patients *(13–15)*. As a result, it has been recommended that monopronuclear oocytes are rechecked for multiple pronuclei within a few hours *(16)* and cultured for biopsy to confirm diploidy or to the blastocyst stage for transfer.

In normally fertilized oocytes, the two pronuclei come together, the chromosomes arrange themselves along a common spindle and syngamy occurs. The centrioles pull the chromatids apart, a furrow appears between the two poles and the zygote cleaves into a two-cell embryo at a mean of 35.6 hours post-insemination *(1,17)*. Evidence from the distribution of surface markers on the human oocyte and embryo confirm observations in other mammalian embryos that the first cell division is meridional, with the polar bodies marking one pole (reviewed in *(18)*), and the second cell division involves a meridional division for one blastomere and an equatorial division for the other *(9)*. Early entry into the first cell division has been used as an indicator of embryo viability. Selection of embryos for transfer on Day 2 from the cohort that had undergone early cleavage by 25 hours postinsemination resulted in higher pregnancy rates *(19,20)*. Sakkas *et al.* *(20)* postulated this increase in viability in early cleaving embryos was due to intrinsic factors regulating cleavage within the oocyte or embryo rather than the timing of fertilization.

On Day 2, embryos are at the four-cell stage of development by 45.5–45.7 hours postsperm insemination, and three days after fertilization are at the eight-cell stage by 54.3–56.4 hours *(1,17)*. The individual cells of the embryo may be asynchronous in their cell division resulting in embryos with uneven cell numbers. Between the four-cell and the eight-cell stage, the transition from maternal to embryonic gene expression occurs; therefore, during the first 48 hours after sperm insemination the embryo primarily relies on maternal transcripts rather than its own activated genome *(21)*.

A morphological criterion used to assess embryo quality throughout human embryology is embryonic fragmentation. Fragmentation is the extrusion of the plasma membrane and subjacent cytoplasm of an embryo into the extracellular region. Fragmentation is not an *in vitro* phenomenon due to compromised embryonic development but appears to be a natural occurrence in human embryos as it is evident in *in vivo* grown embryos *(22)*. The intracellular mechanisms causing fragmentation are not fully known, although it has been speculated that the process may be caused by developmentally lethal defects or apoptotic events *(23)*. Hoover *et al.* *(24)* examined blastomere size and embryo fragmentation as an indicator of embryo viability and pregnancy potential. The authors found blastomere size influenced the developmental potential; however, no correlation was observed with cellular fragmentation. Contrary to this, Giorgetti *et al.* *(25)* suggested that the embryo developmental potential decreased significantly as the number of cytoplasmic fragments increased. Furthermore, Antczak and Van Blerkom *(9)* suggested that the amount of fragmentation an embryo has is not the

determining factor; it is what is being extruded that relates to embryo viability. These authors showed that several regulatory proteins are localized to polarized domains in the oocyte and are asymmetrically distributed to individual cells during developmental progression. A decrease or elimination of these necessary proteins by fragmentation may then lead to blastomere apoptosis. Therefore, the size and distribution of fragments may have different consequences for the developmental competence of the embryo as a whole *(26)*. Small, scattered fragments may be due to imperfect cytokinesis during successive divisions and not specific anomalies, as may be the case with greater localized fragmentation.

Embryo morphology has been associated with chromosomal abnormalities. Almeida and Bolton*(27)* showed that 63.4% of embryos that arrest between the pronucleate and the eight-cell stage are chromosomally abnormal. Embryos exhibiting irregular-shaped blastomeres and severe fragmentation are considered poor quality embryos and show a higher incidence of chromosomal abnormalities (62%) compared to embryos of good quality (22.2%) *(28)*. Fragmentation has been shown to be correlated to chromosomal mosaicism *(29)*. Slow cleaving (two to six cells on Day 3) and rapidly cleaving (nine or more cells on Day 3) embryos show a higher incidence of chromosomal aneuploidy than those embryos showing normal cleavage kinetics (seven to eight cells on Day 3) *(30)*.

On Day 2 or Day 3 an embryo may exhibit blastomeres with more than one nucleus. The incidence of embryos containing multinucleated blastomeres is not uncommon. These embryos are not necessarily degenerate and some are capable of DNA and RNA synthesis *(31)*. However, Munne and Cohen *(32)* showed that 30.4% of arrested embryos were multinucleated. Kligman *et al. (33)* found that 74.5% of multinucleated embryos are chromosomally abnormal compared to 32.3% of nonmultinucleated embryos. Multinucleated blastomeres have been speculated to result from accelerated ovulation induction response *(34)* or cytokinetic failure *(32)* and are indicative of poor development.

During Day 3 postinsemination, the cytoplasm of the embryo may become granular and tiny pits appear (cytoplasmic pitting). There is an increase in cell–cell adhesion early in the morning of Day 3 at the eight-cell stage and loss of definition between individual blastomeres of embryos that are likely to undergo compaction *(35)*. The blastomeres of the embryo undergo a rearrangement process during compaction with the establishment of cell–cell adhesions leading to communication between blastomeres. Desai *et al. (36)* speculate that cytoplasmic pitting and increased cell–cell adherence may be early markers of cytoplasmic activity and potential for embryonic activation, as embryos exhibiting these features were more likely to proceed to the next stages of preimplantation development—the morula and blastocyst stages.

The embryo is termed a morula approximately four days after fertilization when it has undergone compaction, the formation of tight junctions and gap junctions and polarization of the blastomeres, resulting in communication between blastomeres and segregation of two cellular populations of inside and outside cells *(37)*.

Once a cavity forms within the morula it is termed a blastocyst *(38)*. Cavitation involves the formation of the blastocoele, the fluid filled cavity necessary for blastocyst formation. Wiley*(39)* showed that Na^+/K^+ ATPase, located on the basolateral membrane, pumps sodium out of the cell and into the intercellular spaces and water passively follows, thereby forming the blastocoele cavity. As cavitation proceeds, the two populations of cells formed by polarization of the blastomeres during compaction become (i) the trophectoderm that forms extraembryonic tissue; and (ii) the inner cell mass that forms the embryo lineage *(39)*.

In vitro blastocyst formation occurs between Day 5 and 7 postinsemination *(40,41)*. Blastocysts that occur on the respective days do not appear morphologically different, although their growth rate differs, nor do they have significant differences in hCG secretions, the latter suggesting comparable mature trophectodermal tissue *(40)*. The blastocyst has several different appearances depending on its development *(42,43)*. When a cavity is apparent and the inner cell mass and trophectoderm are distinct, the embryo is termed an early blastocyst. The blastocyst begins to increase in size, termed expanding blastocyst, until it has fully expanded. The expansion of the blastocyst causes thinning of the zona pellucida. The zona pellucida is an acellular glycoprotein coat that surrounds oocytes and embryos and is important during fertilization and early preimplantation development as it keeps the blastomeres of the embryos together when there are no intercellular junctions. The zona pellucida thins with the growing blastocyst until it ruptures and the blastocyst begins to herniate

through the zona pellucida in a process called hatching. This occurs on approximately Day 6 or 7. When the blastocyst fully escapes from the zona pellucida it is termed a hatched blastocyst.

A morula may become vacuolated and appear to be cavitated. The vacuoles do not appear to be lined with the trophectoderm or inner cell mass *(40)*. These embryos do not hatch from the zona pellucida, degenerate with ongoing culture (Day 8 or 9) and have significantly less cells than true blastocysts *(41)*. These embryos are termed vacuolated morulae and should be distinguished from blastocysts.

Blastocysts can appear morphologically similar but may contain significantly different cell numbers *(38,41,44–47)* and have a different ability to hatch from the zona pellucida *(46)*. Blastocyst cell numbers may be correlated with embryo viability. Hardy *et al.* *(44)* found expanded blastocysts on Days 5, 6 and 7 had an average of 37.9, 40.3 and 80.6 trophectoderm cells and 20.4, 41.9 and 45.6 inner cell mass cells, respectively, using a simple culture medium. The authors also observed that the total cell numbers were lower in morphologically abnormal blastocysts and blastocysts arising from abnormally fertilized zygotes. Van Blerkom *(46)* found that the stage-specific differentiation of the cells and, in particular, the inner cell mass, may be a function of age of the embryo rather than the overall number of cells that constitute the embryo. The distribution of the cells between the inner cell mass and trophectoderm also affects embryo viability *(44)*.

Therefore, embryo morphology and growth rate are determinants of embryo quality. The overall embryo quality differs markedly and this variation is evident in embryos produced *in vivo (48)*. Patients generally do produce a similar embryo quality from cycle to cycle independent of maternal age. Embryo quality may be an inherent feature of the female and probably depends in part on oocyte maturity *(49)*. There is a decline in embryo quality in embryos generated from oocytes from ageing women. Janny and Menezo *(50)* speculate that this may be due to increased chromosomal abnormalities, the role of maternally inherited products from the oocyte, time of genomic activation, and the temporal pattern of gene expression during the initial embryo development. Munne *et al. (51)* have also shown that embryos that appear morphologically normal contain chromosomal abnormalities and the frequency of these abnormalities increases with maternal age.

Human embryo culture media and culture conditions

Culture conditions for human embryos have evolved over the past decade; significantly increasing the viability of *in vitro* fertilized embryos. Improvements in culture media and the physical environment in which embryos are cultured have resulted from an increased understanding of both the physiology of the embryo and the environment of the oviduct and uterus. Conventional media for culture of human embryos to Day 2 or Day 3 postinsemination were simple media such as T6 *(52)*, HTF *(53)* and EBSS *(54)* containing a serum additive. Development to the blastocyst stage, however, was limited *(55,56)* because these simple media lack important regulators of embryo development. Co-culture of human embryos with various somatic cell monolayers with more complex media and serum additives was shown to support development of embryos to the blastocyst stage *(57–60)*.

Co-culture of human embryos with somatic cells has been reported to improve blastocyst development, decrease fragmentation and/or improve pregnancy and implantation rates *(57,61–63)*, particularly for patients with repeated IVF failures *(61,64,65)*. Somatic cells may benefit embryo development by providing trophic factors and/or by modifying inhibitory media components *(66–68)*. There is not general consensus, however, that co-culture systems improve pregnancy rates *(46,60,69)*. Co-culture is time-consuming with an associated risk of pathogen transmission.

A greater understanding of both the physiological requirements of the embryo as it develops from the zygote to the blastocyst stages *in vitro* [reviewed in *(70,71)*], and the composition of oviduct and uterine fluids *(72–74)* has led to the more recent development of stage-specific or "sequential" complex media for extended culture. The first of such media was G1/G2 *(75)* which was recently modified, resulting in increased pregnancy rates after blastocyst transfer *(76,77)*. Currently, conditions employed for human embryo culture have been largely based on studies using mouse, rabbit and hamster models. Due to the restricted use of human embryos for research purposes, there is a general lack of prospective randomized clinical trial data relating to the effects of variable culture conditions on embryo viability.

The most abundant chemical constituents of

culture media (next to water) include solubilized ions. Ionic components detected in oviduct and uterine fluids *(72)* are present in formulations of both simple and complex media that support human embryo cleavage and blastulation *in vitro*, yet vary markedly in comparative concentration *(78)*. Little evidence exists as to the significance of single ions on the development of human embryos. Many reports implicate the presence and variable concentrations of most of the dissolved salts [e.g. potassium *(79,80)*] in regulation of mouse embryo growth. Only the putative inhibitory effects of inorganic phosphate ions have been investigated clinically *(81)* with contradictory pregnancy results *(82,83)*. The subtle, integrated effects of ions may be better assessed with simultaneous optimization of concentrations, e.g. KSOM medium *(84,85)*, which was recently reported to enhance human blastocyst formation *(86)*.

Irrespective of concentration, ions should be solubilized in injection-quality, filter-purified and toxicity-tested water *(87)* to minimize the risk of biological contamination and to perhaps improve long-term outcome *(88)*. Together with purified water, ionic constituents contribute largely to the osmolality of a medium (or osmotic pressure imparted by dissolved particles)—a testable physical parameter *(89)*. Media that range from 250–290 mOsmols can satisfactorily support mammalian embryo development *(90,91)*. Deviation from this range and especially above 300 mOsmols *(92,93)* can induce deleterious changes in cell volume and hydration resulting in the compensatory uptake of organic osmolytes *(94,95)*.

Protein sources in culture medium, aside from playing a major role in preventing embryo adhesion, may also act as organic osmolytes and pH buffers (reviewed in *(78)*). Filtered, heat-inactivated maternal serum, a traditional additive with undefined, variable composition *(96,97)*, was found to have no effect on *in vitro* fertilization (IVF) pregnancy rates when compared to media without protein supplementation *(98)*. Since then, several attempts have been made to compare fractionated serum proteins to whole sera with equivocal results with respect to embryo quality and development *(99–108)*. Presently, there are several risk factors associated with supplementation of culture media with patient serum such as potential viral transmission *(99)* and the negative effects on embryo development of sera from various subgroups of infertile women *(109,110)*. Other negative effects have included impaired blastocyst development *(55)*, possible trophectodermal *(111)* and mitochondrial deterioration *(112)* and metabolic perturbations *(113)*. More chemically defined alternatives to human sera include purified albumin *(99)*, recombinant serum albumin (synthesized by bacteria) *(114)* and glycosaminoglycan molecules *(115)*.

Media with conventional ionic and protein supplements vary little in their composition of the main energy substrates pyruvate, glucose and lactate which regulate mammalian embryo metabolism in a stage-specific manner *(70)*. Glucose, however, has been excluded from some media formulations *(81, 105)*. Only a small number of studies of human embryos exist that confirm the mammalian evidence suggesting that pyruvate is a requirement to support cleavage and blastulation *(116,117)*. However, there is a shift in preference from pyruvate to glucose with the onset of embryo compaction *(118,119)*. There is little evidence on the effects of energy substrates and their concentrations in IVF culture media formulations on embryo viability. Sequential media were designed to vary in concentrations of carbohydrates to more adequately reflect the difference in composition of fluid components in the oviduct and uterus *(120)*.

Amino acids are found in the fluids of the human reproductive tract *(72,73)*; however, there has been minimal research on the effects of amino acids on the human embryo. Studies in other mammalian species have found amino acids to be important regulators of embryo development [for review see *(78)*]. Glutamine (1 mM) was found to significantly increase development of human embryos to the morula and blastocyst stages and increase energy metabolism *(121)*. Taurine *(94)* and glycine *(95)* have been shown to act as osmolytes and therefore may help to minimize the stress induced by osmotic fluctuations. Interestingly, isoleucine (0.2 mM) and phenylalanine (0.1 mM), both constituents of Eagle's essential amino acids *(122)*, were found to inhibit cleavage and implantation of human embryos *(123)*. Based on mouse and hamster embryo studies, sequential media for human embryos typically contain glutamine, taurine and Eagle's nonessential amino acids for development from Day 1 to Day 3 postinsemination, and glutamine and Eagle's essential amino acids for culture to the blastocyst stage *(75)*.

Vitamins are key components of cellular metabolism and have been shown to have significant effects on embryo quality during culture of rabbit, mouse and hamster embryos *(124–126)*. Despite the presence of B-group vitamins in recently developed sequential media for the development of human

embryos from Day 3 to Day 5, there is no information as to the effects of these vitamins on the human embryo. Ascorbic acid (vitamin C) had no significant effect on embryo development or morphology up to Day 2 or 3 postinsemination *(127)*.

The addition of growth factors such as leukaemia inhibitory factor (LIF) *(128)*, epidermal growth factor (EGF) *(129)* and insulin-like growth factor-I (IGF-I) *(130)*, and the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) *(131)*, has been shown to increase development of human embryos to the blastocyst stage. Furthermore, IGF-I *(130)* and GM-CSF *(131)* stimulated development of the inner cell mass. Thus, further studies are required to determine whether growth factors and cytokines have a significant impact on embryo viability.

EDTA and transferrin have also been added to many media for their potential function as chelators of metal ions. Careful consideration should be given, however, to the addition of EDTA to media for blastocyst development as EDTA has been shown to reduce glycolytic activity and thus inhibit blastocyst development in the mouse *(132)*.

Human embryos have typically been cultured individually in large volumes of media (usually one millilitre). Studies in the mouse have found that embryo density has a significant effect on cleavage rate and blastocyst development *(133,134)*, possibly due to embryo-derived trophic factors. Culture of human embryos in groups for up to 48 hours post-insemination was associated with an increased rate of cleavage *(135,136)*; however, the effects on pregnancy rates have been variable *(135–138)*. Rijnders and Jansen *(139)* reported that culture of human embryos to the blastocyst stage in either reduced volumes of media and/or groups, had no effect on pregnancy rates; however, the culture media was not sequential and the sample size was low.

Further improvements could be made to the formulation of media for human embryo culture through determination of the effects of individual medium components on embryo development and viability. The physical conditions under which embryos are cultured also have a significant impact on viability. The majority of culture systems for human embryos use a bicarbonate/CO_2 buffered medium to maintain a physiological pH of 7.2–7.4. Embryos are typically cultured in an incubator at a gas atmosphere containing 5% CO_2 *(76,139,140)*. According to the Henderson-Hasselbalch equation, the combination of 25 mM sodium bicarbonate in the culture medium and a 5% CO_2 gas atmosphere, results in a pH of 7.45 at sea level. Thus, it would be prudent to culture embryos at a slightly elevated concentration of 6% CO_2 *(123)*, which theoretically results in a pH of 7.37. The optimal concentration of CO_2 for culture of human embryos is yet to be determined. An N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffered medium is often used to maintain a pH of 7.3–7.4 when embryos are exposed to atmospheric oxygen tensions for an extended period of time. It is important to consider, however, that exposure to atmospheric CO_2 and a reduced concentration of HCO_3^- may affect the embryo's ability to regulate its intracellular pH, specifically acidosis, via the HCO_3^-/Cl^- exchanger *(141)*.

The oxygen tension in the reproductive tract of mammalian species has been reported to be lower than atmospheric O_2, ranging between 1.5% and 8% *(142,143)*. Recent evidence indicates a low pO_2 in the human vaginal epithelium *(144)*. Culture of mammalian embryos at low concentrations of O_2 (5%–7%) is thought to minimize the formation of embryotoxic reactive oxygen species; however, studies on human embryos are few. Culturing human embryos at a reduced oxygen tension (5%) for up to 46 hours following insemination had no effect on cleavage rates or subsequent pregnancy rates *(140)*. Culture of frozen-thawed pronuclear stage embryos for four days at a reduced oxygen tension (5%) did not affect blastocyst development but did significantly increase total cell number compared to culture at 20% O_2 *(145)*. Thus, it may be prudent to culture human embryos at a reduced oxygen tension.

Further optimization of human embryo culture systems is needed to gain a fuller understanding of the specific requirements of the human embryo for regulatory support.

Selection of embryos for transfer

One of the most difficult aspects of assisted reproductive technology (ART) is the determination of which embryos are most suitable for transfer into the uterus. Two factors requiring consideration include the choice of embryos with the best developmental competence and the risks of multiple pregnancy associated with the number of embryos transferred. The development of technological advances such as micromanipulation and a wealth of experience in embryo culturing techniques have resulted in an

increase in embryo implantation potential. Numerous criteria have been suggested to optimize the selection process. These include: the rate of embryo development; blastocyst development; pronuclei expression and nucleoli orientation; ovarian/follicular vascularity; noninvasive assessment of metabolic products of embryos during development; preimplantation genetic diagnosis; and morphological assessment. Embryo scoring techniques have been developed to aid in evaluating the potential of embryo implantation.

Morphological assessment has been employed for many years as a tool in determining which embryos display the greatest pregnancy potential *(146)*. Embryos are scored based on cell number, fragmentation pattern and extent, cytoplasmic pitting, blastomere regularity, presence of vacuoles and blastomere expansion *(36,147–151)*.

Fragmentation is one of the most common morphological features used in assessing embryo quality. Grading systems have gauged embryo quality based on the percentage of fragmentation observed within the embryo. Low implantation rates have been reported from embryos with 10%–50% fragmentation on Day 2 of development*(25,152)*. Not all fragmentation appears to be detrimental to embryo development but the pattern of fragmentation has a profound effect on the embryo's developmental potential. Large fragments formed at the two-cell to four-cell stage appear more detrimental due to the depletion of essential organelles such as mitochondria, or structures such as pinocytotic caveolae, involved in exogenous protein uptake *(26)*. The presence of small fragments does not appear to effect developmental rates to the same degree as large fragments. The formation of small fragments may represent incomplete cytokinesis. Implantation rates are similar between embryos without fragmentation and those with moderate fragmentation. Antczak *et al. (9)* compared the relationship between blastomere fragmentation and the effect on the distribution of regulatory proteins. The findings demonstrated that certain patterns of fragmentation might result in partial loss of certain regulatory proteins from specific blastomeres, resulting in compromised development if fragmentation occurs during the one-cell or two-cell stages. Correlation between fragmentation and apoptosis is not clear but fragmentation may be an initiator of apoptosis if regulatory proteins are altered.

Embryo development rates were initially used in scoring for embryo's developmental competency*(17)*. Early cleavage of fertilized oocytes to the two-cell stage is used for its prognostic value in determining embryos for transfer *(19)*. A study by Giorgetti *et al. (25)* involving single embryo transfers, concluded that the use of embryo scoring based on cleavage rate and morphology was advantageous in maximizing pregnancy rates. One of the shortcomings of early cleavage as a selection criterion relates to oocyte maturity. Immature oocytes may fertilize later than mature oocytes under standard IVF culture conditions. Oocytes selected for intracytoplasmic sperm injection (ICSI) are biased due to maturation status. Sakkas *et al. (20)* concluded that early cleavage rate was not entirely influenced by timing of fertilization but is more likely influenced by intrinsic factors within the oocyte or embryo. The expression of human leukocyte antigen G (HLA-G) has been demonstrated to correlate with mRNA expression and improve cleavage rates *(153)*. Embryos that have undergone early cleavage may be less likely to experience critically low reserves of maternal mRNA prior to embryonic genome activation *(154)*.

Cell stage at the time of transfer has become a significant factor in determining which embryos have the greatest potential for implantation. Embryonic genome activation occurs between the four-cell and eight-cell stages of preimplantation development*(21)*. Delaying embryo transfer to Day 3 of development allows a selection of embryos undergoing embryonic activation.

In an attempt to further refine selection criterion based on morphology, zygote scoring of pronuclei was investigated. Scott *et al. (155)* outlined scoring systems based on the alignment of nucleoli at the junction of the two pronuclei and the appearance of the cytoplasm. These systems have been refined over time to encompass embryo morphology and development rates and now include nucleoli alignment, appearance of cytoplasm and the incidence of blastomere multinucleation.

The scoring system, often classified as a "Z" rating, for zygote, records a number of crucial phases of development. The first record of pronuclei alignment or appearance of "touching" at 16–18 hours postinsemination relates to activation of the oocyte by the introduction of the spermatozoon. The sperm-derived centriole and the microtubules arising from it are responsible for alignment of the pronuclei. If this fails to occur, developmental potential is limited*(29)*. Pronuclei are often slightly different in size but large differences have been associated with chromosomal defects such as aneuploidy *(7)*. Another aspect of

"Z" scoring relates to size, number and distribution of nucleoli. Nucleoli are the sites where pre-rRNA is synthesized. Following fertilization, rRNA synthesis resumes and the nucleoli reform and grow. It is presumed that "Z" scoring allows observation of the resumption of rRNA synthesis *(155)*. Zygotes with three to seven even-sized nucleoli per nucleus appear to give rise to embryos with greater developmental potential. Pronuclei scoring used in association with embryo development and morphology may offer a technique for determining which embryos would benefit from prolonged *in vitro* culture *(156–158)*, particularly as the score has been related to the ability to continue development to the blastocyst stage *(3)*.

Extended embryo culture to the blastocyst stage was proposed as a possible solution to the risks of multiple pregnancy. Determination of which embryos survive to the blastocyst phase was considered the most vital criterion for selection of embryos with the greatest implantation potential. Blastocyst culture addresses the issue relating to endometrial asynchrony, uterine hostility associated with early cleavage-stage embryos, and the assumption that all embryos have equal implantation potential. Selection of blastocyst-stage embryos may allow for the transfer of a single blastocyst.

Blastocyst culture has developed significantly over the past few years but is still fraught with the inherent problem of zygote development potential *(159)*. Only half of all zygotes have the potential to develop to the blastocyst stage. Aneuploidy can be used as an explanation for approximately half of the embryos failing to develop to the blastocyst stage *(160)*. Extended culture to the blastocyst stage does not eliminate those embryos displaying chromosome abnormalities *(160)* as 40% of embryos displaying normal morphological development to blastocyst are aneuploid.

Blastocyst development occurs between Day 5 and 7 postinsemination. Menezo *et al. (161)* observed that the transfer of embryos at the compacting morula stage resulted in poor pregnancy rates. This is thought to be associated with the fragile nature of the embryo at this phase of development. Pregnancies have been noted from the transfer of blastocysts ranging from Day 5 to Day 7 of development. Shoukir *et al.(162)* suggested that "good" quality blastocysts not only displayed well expanded blastocoelic cavities and well-defined inner cell masses but also had attained this stage by Day 5 or 6. A scoring system for blastocyst development was first described by Dokras *et al. (41)*. This system uses three grades for blastocyst classification based on the timing of cavitation, cavity formation and inner cell mass definition and trophectoderm distinction. Scoring systems have undergone further refinement and now include blastocoele volume, zona thinning and blastocyst hatching *(120)*. Scholtes *et al. (163)* suggested the success of blastocyst culture techniques depends primarily on the number of oocytes retrieved and not maternal age. Others have also noted that there is a reduction in the number of blastocysts formed in cases of male infertility *(42,164,165)*.

Assessment of embryo metabolism presents another potential technique for viable embryo selection. Gardner *et al. (70)* suggested that glucose uptake and metabolism might be used to predict the embryos most suitable for transfer. The noninvasive measurement of glucose and pyruvate uptake by human embryos *(166)*, the measurement of ATP and ADP levels *(167,168)*, the expression of EGF, transforming growth factor alpha and EGF receptor *(169)*, extracellular matrix protein production *(170)*, the role of pregnancy-specific β-1 glycoprotein *(86,171)*, production of human chorionic gonadotrophin (hCG) and HLA-G and pregnancy-specific β-1 glycoprotein *(86)*, and expression of IGF *(172)* have all been suggested as having potential value in predicting those embryos with high implantation competency. Jones *(159)* concludes that pregnancy; specific β-1 glycoprotein appears to be the biochemical factor with the greatest potential to act as a prognostic indicator of blastocyst viability. The use of biochemical factors as predictive markers in embryo selection is limited to those techniques that use noninvasive assessments or measurements. Further investigation of such markers is required. Follicular vascularity has also been suggested as another tool in determining follicles containing oocytes with greater developmental potential. Nargund *et al. (173)* concluded that a statistical correlation does exist between follicular peak systolic velocity, the ability to retrieve an oocyte and morphological development. Van Blerkom *et al. (174)* suggested an association between intrafollicular oxygen content and perifollicular vascularity, which may provide a useful indicator in oocyte developmental potential. Defects in chromosomal number, spindle organization and cytoplasmic structure have been observed in embryos developing from oocytes retrieved from hypoxic follicles. Colour Doppler imaging has been used as a predictive tool in analysing which preovulatory

follicles may contain oocytes with the potential to develop to normal embryos *(175,176)*. The predictive value of perifollicular blood flow on embryo development remains to be determined but it may offer additional information useful in predicting embryos with high implantation potential.

Embryonic developmental failures have been associated with cleavage arrest and chromosomal abnormalities. Aneuploidy is commonly associated with embryo arrest *(51)*. Preimplantation genetic diagnosis offers another useful technique to eliminate chromosomally abnormal embryos with little to no developmental potential prior to transfer. Sex selection of embryos for couples with sex-linked genetic disorders offers the possibility of eliminating carrier embryos prior to transfer.

Numerous embryo scoring systems have been designed to assist in determining which embryos have the greatest developmental potential. Regardless of the many criteria proposed to aid in the selection process, no single criterion has been identified which offers a significant benefit over the others. The majority of embryo selection systems are based on a combination of criteria, including morphology, cleavage rate and embryonic genome activation. The use of blastocyst culture, first suggested as the most useful embryo selection process, does not take into consideration reduced oocyte numbers and embryo production due to maternal age and ovulatory defects. Nor does it overcome the problem of identifying embryos with potential genetic abnormalities. Racowsky *et al. (177)* suggested an embryo selection process which compensated for maternal age and etiology. This selection process incorporates the number of eight-cell embryos available for transfer on Day 3 postinsemination. Patients with greater than three morphologically "good" embryos are encouraged to undergo Day 5 (blastocyst) embryo transfer. This process not only attempts to address endometrial asynchrony but also to reduce the risks of multiple pregnancy due to the transfer of fewer embryos.

Preimplantation genetic diagnosis (PGD) is becoming a useful adjunct in embryo selection with the development of additional DNA probes, particularly in cases of advanced maternal age. DNA fingerprinting may prove to be a significant tool in finally assessing which of the simple selection criteria are beneficial. The current practice of transferring multiple embryos precludes the identification of which embryo is responsible for the pregnancy and DNA fingerprinting may provide conclusive evidence as to which embryo is responsible for each live birth. Although a long-term assessment technique, eventually embryologists may be able to quantify, retrospectively, the criteria that are essential in determining the "best" embryos for transfer.

Despite all the advances in determining embryo developmental competence, no consensus has been reached on how many embryos to transfer. Multiple pregnancy rates remain high with recommendations of three or more embryos for transfer, depending on maternal age, still being promoted *(178)*. One of the potential benefits of refining embryo selection criteria is to reduce the number of embryos for transfer and thus the high multiple pregnancy rates.

Embryo transfer

The embryo transfer procedure should be considered as important to the success of ART as embryo quality and uterine receptivity. However, embryo transfer is perhaps the least understood link in the chain of procedures. Embryos from the one-cell stage (pronucleate embryos or zygotes) to the blastocyst stage of development may be transferred to either the fallopian tube or the uterus.

The fallopian tube can be cannulated from either the fimbrial end (orthograde transfer) or the uterine end (retrograde transfer) *(179)*. The surgical techniques of laparoscopy or laparotomy (orthograde transfer), or the nonsurgical techniques of transcervical or transvaginal cannulation (retrograde transfer) under ultrasound guidance, hysteroscopic guidance or by tactile sensation, can be used to cannulate the fallopian tube *(180)*. Laparotomy is no longer needed for tubal catheterization, as the procedure can be performed successfully via laparoscopy, using video guidance *(180)*. However, laparoscopy usually requires the patient to have a general anaesthetic and the abdomen insufflated with CO_2 *(181)* with attendant risks and side-effects. For these reasons, laparoscopic intrafallopian transfer has been attempted with success under local anaesthesia, but has not received wide acceptance *(181,182)*. Laparoscopic transfer has fallen out of favour since the introduction of transvaginal ultrasound-guided oocyte retrieval, which is usually performed with no anaesthesia, with or without light sedation. Cannulation of the fallopian tube via the cervix under ultrasound guidance, hysteroscopic guidance or by tactile impression, without general anaesthesia has

been performed with success for transcervical zygote intrafallopian transfer (ZIFT), tubal embryo stage transfer (TEST), tubal embryo transfer (TET). However, complications such as uterine anomalies, a retroverted uterus, or unsuspected tubal cornual obstruction may make it impossible to regularly cannulate the fallopian tube *(183)*. Transcervical transfer to the uterus can then be undertaken but it is possible the endometrium may have already been traumatized during the initial attempts to cannulate the fallopian tube with a consequent reduction in the chance of implantation *(184)*. There are also side-effects of the procedure including the possibility of flushing the embryos from the fallopian tube if injection flow rates are too high *(185)*, tubal perforation, vasovagal faintness and pelvic discomfort in some (less than 10%) patients *(179)*.

Zygote or embryo transfer into the fallopian tube is not possible in all patients, and in order to minimize the risk of ectopic pregnancy, should only be performed when the fallopian tube is patent and when the patient has had no previous history of pelvic inflammatory disease, ectopic pregnancy, or tubal surgery *(179,184,186)*. The fallopian tube transfer procedures include pronucleate stage transfer (PROST) in which pronucleate-stage embryos are transferred to the fallopian tube, ZIFT in which pronuclear to early cleavage-stage embryos are transferred to the fallopian tube, and TET or TEST in which early cleavage-stage embryos are transferred to the fallopian tube.

PROST has been used for patients in whom the etiology of infertility is unexplained or due to moderately severe male factor, antisperm antibodies or endometriosis. Similarly, the techniques of ZIFT, TET and TEST have been used for patients with male factor or immunological infertility and, in addition to confirming successful fertilization, offer the further possibility of eliminating those zygotes that fail to undergo the first cleavage divisions.

Until recently, one of the disadvantages of transferring early zygotes was the inability to select the most viable zygotes from a large cohort for transfer. However, high pregnancy and implantation rates have recently been reported for PROST when zygotes are selected for transfer according to certain pronuclear morphological features *(155)*. Early reports comparing embryo transfer to the fallopian tube to transfer to the uterus reported significantly higher implantation, pregnancy and birth rates when embryos were transferred to the fallopian tube *(187–190)*. However, others have reported no benefit when transferring embryos to the fallopian tube instead of the uterus *(191–196)*.

The majority of embryo transfers are currently carried out by nonsurgically cannulating the uterine cavity via the cervix (transcervical transfers). However, it is also possible to transfer embryos to the uterus using surgical techniques; ultrasound-guided perurethral transvaginal embryo transfer *(197)*; or transmyometrial transfer *(198,199)*. These methods have been used for patients with nonpatent tubes when anatomical abnormalities of the uterus or severe cervical stenosis would predict that cannulation of the cervical canal would be difficult or impossible *(180)*.

Transmyometrial embryo transfer is widely practised in Asia and pregnancy rates are reported to be high *(198)*. However, in a randomized prospective trial, no benefit could be demonstrated for transmyometrial transfer over transcervical transfer and pregnancy rates were low *(199)*. This difference may be due to anatomical features of the cervix in oriental women. A more compressed cervical canal compared to those from other races is frequently observed, but further studies need to be carried out to evaluate this *(200)*.

Transcervical embryo transfer is a rapid and easy technique, and does not require analgesics or anaesthetics *(180)*. Disadvantages include the technical difficulty encountered in patients with cervical stenosis *(199)*, the risk of infection from the introduction of microorganisms into the endometrial cavity *(179)*, and release of prostaglandins that may cause myometrial contractions and loss of embryos into the fallopian tube or vagina *(179)*.

To perform an embryo transfer, preparation of the patient is required. This includes positioning the patient, introducing the speculum, cleaning the cervix and manipulating the uterus.

The dorsal lithotomy position is most commonly used for embryo transfer, especially for patients with an axial or retroverted uterus, whereas for patients with an anteverted uterus, the knee–chest position has been recommended to eliminate expulsion of embryos from the uterus *(201)*. However, there is no convincing evidence that patient position affects the outcome of embryo transfer *(202–204)* and it is recommended to choose a position most comfortable to both patient and clinician.

A bivalve speculum is then introduced gently into the vagina to expose the cervix. Manoeuvring the speculum can improve cervico-uterine alignment to

allow easier access by the catheter *(200)*. Further manipulation can be achieved by passive bladder distension which has been reported to result in significantly higher pregnancy rates than when patients have an empty bladder *(205)* and is a requirement if abdominal ultrasound monitoring of catheter placement is to be employed. The uterus can also be straightened artificially by using a tenaculum or cervical suture; however, this may stimulate uterine junctional zone contractions and lead to implantation failure *(206)* or the transportation of the embryos into the fallopian tube, increasing the risk of ectopic pregnancy *(207)*. For patients with cervical stenosis where passage of the catheter is extremely difficult, cervical dilatation can be performed. It has been recommended that cervical dilatation be performed at the time of embryo transfer *(208)* rather than at the time of oocyte retrieval *(209)* as it does not appear to affect pregnancy rates.

The vulva and vagina require no special preparation but the cervix can be cleaned by swabbing, vigorous flushing, or aspiration to remove excess mucus and there is no clear consensus as to which is the best method. Some favour complete aspiration of cervical mucus *(210)* whereas others have demonstrated improved results when the cervix is vigorously flushed with culture medium to remove mucus *(211)*. As yet, no controlled, randomized studies have been carried out to evaluate the requirement of removing mucus prior to embryo transfer.

To gain a better understanding and knowledge of each patient's anatomy, a "mock" embryo transfer can be performed. A mock transfer can take place either in a cycle prior to the treatment cycle *(201,212)* or just prior to the real embryo transfer procedure *(213)*. Performing a mock transfer offers many potential advantages: the most suitable catheter can be chosen for each patient to facilitate atraumatic transfer; the direction of the uterus can be assessed and the length of the uterus can be measured *(213)*. The main disadvantage of performing the mock transfer before the treatment cycle is that the uterus is mobile so it is possible that the direction of the uterus may be different on the day of the real embryo transfer *(213)*. Mock embryo transfer has been shown to minimize the problems associated with embryo transfer and to improve pregnancy and implantation rates *(212)*.

Medications such as anaesthesia, uterine relaxants or prophylactic antibiotics and corticosteroids have been given during the embryo transfer procedure. General anaesthesia as a routine for embryo transfer has not proven to significantly improve pregnancy rates *(202)*. However, general anaesthesia is sometimes required if the embryo transfer procedure is extremely difficult. Care should be taken as to the choice of anaesthetic agent used as it may affect results *(214)*. Tranquillizers such as diazepam have also been used to reduce patient anxiety and promote ease of transfer *(202,212)*. However, it has become common practice to withhold medication except in the case of very difficult transfers. Instead, patient reassurance by staff members familiar to her, previous experience with mock embryo transfer and a physician performing the procedure who is known to the patient, have appeared to be sufficient to achieve patient relaxation and improve ease of procedure.

Prostaglandins may adversely affect outcome following embryo transfer due to their action in stimulating uterine contractions. Prostaglandin inhibitors such as ibuprofen *(215)* or indomethacin *(201)* have been used to inhibit uterine contraction but have not had any beneficial effect on pregnancy rate *(202)*. Similarly, administration of the smooth muscle relaxant, glyceryl trinitrate, has no affect on pregnancy rate *(216)*.

Microbial contamination of the embryo transfer catheter tip is correlated with a significant reduction in pregnancy rate *(217,218)*. Prophylactic antibiotics administered at the time of oocyte retrieval significantly reduce the incidence of positive microbial cultures from embryo transfer catheter tips 48 hours after antibiotic administration *(219)*. However, no controlled, randomized studies have been undertaken to investigate the effect on pregnancy rates. One of the many protective functions of the zona pellucida surrounding the early cleavage-stage embryo is in reducing contact with microorganisms and immune cells. Zona-manipulated embryos when transferred to the uterus, are potentially at risk of exposure to these cells and for this reason, low-dose immunosuppression with corticosteroids has been advocated *(220)*. However, the effectiveness of this immunosuppression and its effect on pregnancy outcome has not been investigated in any systematic way.

There are over 50 different catheters available commercially for clinical embryo transfer and several studies have been undertaken to compare different catheters *(221–227)*. Catheters are classified according to their tip, flexibility, presence of separate outer sheath, location of the distal port (end- or side-loading), degree of stiffness and malleability, memory, thickness and length. Catheters are generally manu-

factured from nontoxic plastic materials but have different sterilization processes that may affect the relative toxicity and handling procedures *(200)*. There is little difference in concept and technology between embryo transfer catheters and so it is not surprising that there is no clear consensus as to the one catheter that is superior. However, soft embryo transfer catheters are used most frequently as they produce superior results *(221,222)* and are easy to use in all but the most difficult transfers *(222)*. The main characteristic required of a transfer catheter is the ability to manoeuvre into the uterine cavity while causing no trauma to embryos and endometrium.

There is as yet no consensus as to the depth of placement of ET catheters within the uterus. However, contact between the catheter and the uterine fundus stimulates junctional zone contractions that may be responsible for relocating intrauterine embryos, and so contribute to embryo transfer failure or ectopic gestation *(228)*. High-frequency uterine contractions on the day of embryo transfer have been found to decrease clinical and ongoing pregnancy rates as well as the implantation rate, possibly by expelling embryos from the uterine cavity *(229)*. Others have reported that there is no relationship between the site of embryo deposition in the uterus monitored by ultrasound (measured as distance from fundus) and the pregnancy outcome *(230)*. However, all embryos were deposited at least one to two millimetres from the fundus and no mention was made as to whether the fundus was touched by the catheter. Although there is no agreement as to the exact depth of placement of the catheter within the uterus, there appears to be general agreement on the avoidance of touching the uterine fundus *(231,232)*.

In order to assist more accurate catheter placement within uteri of various dimensions *(233)*, ultrasound-guided embryo transfer has been developed instead of relying on clinician "feel". Studies comparing ultrasound-guided embryo transfer to embryo transfer by clinician "feel". failed to demonstrate a significant improvement in pregnancy and implantation rates *(234,235)*, with the exception of a small subgroup of patients receiving a single embryo for transfer *(234)*. However, tactile assessment of embryo transfer catheter placement has been demonstrated to be unreliable *(236)*: in 17.4% of cases the guiding cannula was inadvertently abutting the fundal endometrium; and in 7.4% of cases abutting the internal tubal ostia. More recent studies suggest that an improvement in both the clinical pregnancy rate and the implantation rate can be achieved if ultrasound-guided embryo transfer is used *(221,237)*. Ultrasound confirmation of the retention of the fluid droplet containing the embryos at the site of catheter placement results in improved clinical pregnancy rates *(221)*.

During ultrasound-guided embryo transfer, Woolcott and Stanger *(236)* identified that the transfer catheter embedded within the endometrium in 24% of cases and deposition of the embryos beneath the endometrium (intraendometrial transfers) occurred in 22% of cases. However, this appeared to have no effect on pregnancy outcome. Intentional intraendometrial embryo transfer has been carried out under direct visualization using a CO_2-pulsed flexible hysteroscope *(238)*. A very low implantation rate resulted and it was concluded that the acidifying effect of the CO_2 on the endometrial stroma might have produced a suboptimal environment for early embryonic development.

To minimize the potential for movement and expulsion of embryos following embryo transfer, a fibrin sealant, or biological glue, has been used to attach embryos to the endometrium at the site of embryo deposition *(239,240)*. In a prospective, randomized study, no significant difference in clinical pregnancy rate or ongoing pregnancy rate, but a significant reduction in ectopic pregnancies, was found *(240)*. No follow-up studies have been reported.

At the completion of transfer, the catheter should be examined carefully by the embryologist for retained embryos. Blood, mucus or uterine tissue may attach to the end of the catheter and impede egress of the embryos from the catheter. Blood on the outside of the catheter has been related to poorer results *(241)*. Visser *et al.* *(242)* reported that failure to deliver embryos on the first attempt at embryo transfer resulted in a decrease in pregnancy rate and suggested that retained embryos should be transferred one day later. In contrast, no difference in the clinical pregnancy rate was found in another study when all embryos were transferred in the first attempt compared to when repeated attempts were necessary to transfer all embryos *(243)*.

Historically, bed rest for up to 24 hours following embryo transfer has been advised. Bed rest began as a precautionary measure to try to improve implantation rates, even though there is a lack of evidence as to its benefit. Immediate mobilization of the patient following embryo transfer does not, however, appear to have a detrimental effect on pregnancy outcome

(244–246). It has been shown by ultrasound that standing immediately after embryo transfer resulted in movement of embryo-associated air bubbles within the uterine cavity in only 6% of cases *(247)*. Furthermore, there was no evidence of movement of embryo-associated air outside the uterine cavity and it would appear that standing shortly after transfer plays no significant role in the final position of transferred embryos.

It is difficult to know which embryo transfer protocol to follow to ensure the highest degree of success, as many of the technical aspects have undergone very little scientific evaluation, if any. In fact, little evidence exists to support many of the choices made in this aspect of ART. It is clear, however, that an easy, atraumatic transfer is important with factors such as patient preparation, medication, mock embryo transfer, choice of catheter, transfer technique and bed rest optimized to provide improved outcomes and patient well-being.

Day of transfer

Embryos may be transferred to the patient on Day 1 postinsemination at the zygote stage of development (PROST, ZIFT), on Day 2 as two-cell to four-cell early cleavage-stage embryos (ZIFT, TEST, TET, ET), on Day 3 as six-cell to eight-cell early cleavage-stage embryos (ZIFT, TEST, TET, ET), on Day 4 as morulae (ET), or on Days 5 to 7 as blastocysts of varied morphology (ET).

In the past 10 years, the majority of clinics have opted to transfer embryos at the early cleavage stage of development to the uterus on Day 2 postinsemination, despite the fact that, *in vivo*, the embryo would not pass into the uterus until two to three days later at the morula to blastocyst stage of development *(22, 248)*. Transfer of early cleavage-stage embryos has been the preferred option as it allows confirmation of fertilization and a limited assessment of developmental potential as the embryo undergoes its first few cleavage divisions, while at the same time minimizing the potential compromise to embryo viability posed by prolonged exposure to suboptimal *in vitro* culture conditions. In fact, when *in vitro* culture conditions are significantly compromised, embryo transfer at the zygote stage of development, rather than at the early cleavage stage of development, results in much higher pregnancy rates *(249)*.

Recent advances in the formulation of embryo culture medium and culture conditions has seen a shift in interest toward transfer of later-stage embryos, particularly blastocysts, as high pregnancy and implantation rates have been reported despite the fact that fewer embryos are usually transferred *(77,120, 250–252)*. Transfer at the morula to blastocyst stage of development allows selection of embryos that have demonstrated the potential for continued development under embryonic genomic control *(21)*, although these embryos may not necessarily have a normal chromosome complement *(160)*.

More recently, morphological parameters have been established for embryos at the pronucleate or zygote stage of development that identifies embryos with a high viability *(3,6,19,20,155–158)*. It is difficult to determine which of the various options for day of transfer will result in the highest success rates as very few controlled, randomized comparisons using similar patient populations have been undertaken.

van Os *et al. (253)* were the first to demonstrate that embryo transfer could be delayed from Day 2 to Day 3 without any impact on subsequent pregnancy rates. In fact, they argued that a higher number of viable pregnancies was established following transfer of embryos on Day 3. In a later study, Dawson *et al. (254)* demonstrated that although the pregnancy rate is not different when embryos are transferred on Day 2 or Day 3, the implantation rate is significantly higher on Day 3, indicating that selection of viable embryos is improved with a further day in culture. This was further supported by the finding that fewer embryos miscarried before six weeks of gestation when embryos were transferred on Day 3 *(254)*. A recent study, using one of the newer culture medium formulations without glucose and phosphate, reported a significant improvement in both pregnancy and implantation rates when embryos were transferred on Day 3 rather than on Day 2 *(82)*. In contrast, Ertzeid *et al. (255)*, in a prospective, randomized study, showed no benefit on the implantation rate or the live birth rate in delaying transfer from Day 2 to Day 3.

Similarly, delaying embryo transfer until Day 4 postinsemination results in similar implantation and pregnancy rates to those achieved following embryo transfer on Days 2 and 3 *(256,257)*. In addition, Huisman *et al. (256)* noted that delaying transfer to Day 4 provided the ability to identify embryos with a very high implantation potential. Transfer of cavitating morula stages on Day 4 resulted in a 41% implantation rate per embryo *(256)*. It was suggested that deferring embryo transfer for a few days may provide

the possibility of selecting fewer and better quality embryos for transfer and therefore limit the incidence of multiple pregnancy *(256)*.

Initial attempts at extending culture to the blastocyst stage followed by embryo transfer on Day 5 resulted in disappointingly low pregnancy and implantation rates *(56, 257)*. However, improvements in the culture media and culture conditions for human embryo development in the past few years have resulted in the successful development of viable blastocysts (reviewed in *(159)*). Scholtes and Zeilmaker *(258)*, in a prospective randomized trial, reported no significant difference in pregnancy and implantation rates when embryo transfer was performed on either Day 3 or Day 5. However, deferring embryo transfer to Day 5 allowed the identification of embryos with very high implantation potential. Pregnancy and implantation rates were almost double that recorded for Day 3 transfers when exclusively cavitating embryos were transferred on Day 5. Gardner *et al.* *(77)* using culture media formulated to mimic physiological parameters reported a significantly higher implantation rate when blastocysts were transferred on Day 5 compared to early cleavage-stage embryo transfer on Day 3 (51% and 30%, respectively). They further observed that the implantation rate following blastocyst transfer was not affected by the number of blastocysts transferred, suggesting that high-order multiple pregnancies could be avoided by reducing the number of embryos transferred without a corresponding reduction in the pregnancy rate. Similarly, Milki *et al.* *(252)*, using similar patient populations demonstrated that the implantation and pregnancy rates following blastocyst transfer on Day 5 was higher than following transfer on Day 3. In contrast, in prospective, randomized trials using an unselected population of patients, it has been reported that there is no difference in pregnancy and implantation rates when a similar number of embryos are transferred on either Day 3, Day 4 or Day 5 *(259,260)*. Coskun *et al.* *(259)* suggested that the superiority in selection of any particular embryo stage should be shown by comparing the result of transfer of the best single embryo at any stage in a randomized trial.

Blastocyst development occurs *in vitro* between Days 5 and 7 *(40,41)*. Although several studies have reported that the rate of development to blastocyst affects viability *(162,261)*, others have suggested that in some instances, blastocysts that form as late as Day 7 may have some inherent viability as there is no difference in cumulative hCG secretion by embryos which formed blastocysts from Days 5 to 7 *(41,262)* and transfer of Day 7 blastocysts occasionally results in pregnancies *(161)*. The majority of programmes transfer blastocysts on Day 5 but some programmes have elected to delay transfer to Day 6 to allow for a further element of selection among a cohort of blastocysts *(42,76,263,264)*. Delaying transfers until a time when selection of fully expanded or hatching blastocysts is possible, rather than automatically transferring blastocysts on Day 5, may increase implantation rates by providing a further element of selection *(42)*. Transfer of cryopreserved blastocysts has revealed that pregnancies can be achieved following transfer of blastocysts on Days 5–9 following the LH peak but no pregnancy resulted from transfer of blastocysts as early as Day 4 following the LH peak *(162)*.

One of the advantages of deferring embryo transfer beyond Day 3 is the possibility of performing preimplantation genetic diagnosis. Embryo biopsy is usually performed on Day 3 and successful pregnancies have been established following transfer of biopsied embryos on Day 4 which has allowed a full day for genetic analysis by polymerase chain reaction (PCR) *(265)* or by repeated cycles of fluorescence *in situ* hybridization (FISH) *(266)*. There is also a significant advantage to extending culture to the blastocyst stage of development before performing the biopsy. At this stage, more cells can be biopsied from the extraembryonic trophectoderm for more complex and accurate preimplantation genetic analysis *(262,267,268)*. Preliminary research results on the recovery rate of biopsied human blastocysts are promising *(262,268)* but as yet the procedure has not been performed clinically.

One of the disadvantages of deferring embryo transfer to Day 5 or beyond is that the embryo transfer may be cancelled if no blastocysts develop *in vitro*. Certainly, for some groups of patients, transfer on Day 5 may not be the best option. It has been reported that sperm quality can affect the number of blastocysts developing *in vitro* *(42,164,165)*. In addition, several studies have reported a maternal age-related decline in the number of embryos developing to blastocysts *in vitro* *(50,269,270)*. This finding may be due to the progressive reduction in the number of oocytes retrieved with advancing maternal age *(50)*, as the number of oocytes retrieved is correlated to the number of blastocysts that develop *in vitro* *(42)*. An increase in the number of oocytes retrieved can

ameliorate the negative affect of maternal age *(163)*. Racowsky *et al.* *(177)* suggested that the number of eight-cell embryos on Day 3 should be used as the determinant for the day of transfer as this has been correlated to the number of blastocysts developing *in vitro (42)*. For patients with three of more eight-cell embryos on Day 3, transfer on Day 5 results in a high ongoing pregnancy rate with a significant reduction in the incidence of high-order multiple pregnancies *(177)*. Poor prognosis patients with no eight-cell embryos on Day 3 do not benefit from deferring embryo transfer from Day 3 to Day 5 (33% and 0% pregnancy rate, respectively) *(177)*. It has been hypothesized that the uterus is apparently able to “rescue” some of the suboptimal, slower cleaving embryos and that extending the culture time to Day 5 for these suboptimal embryos, even in optimized culture systems, reduces their viability *(177,271)*.

In conclusion, the introduction in recent years of more subjective selection criteria that are better able to predict viability has resulted in reports of high implantation rates following transfer of embryos from the zygote to the blastocyst stage of development. Although blastocyst transfer has not always resulted in an improvement in pregnancy and implantation rates in the wider population of infertile patients, it appears to be the most likely choice if the number of embryos transferred is to be reduced to one to eliminate multiple pregnancies. As there is a relationship between zygote morphology and embryo morphology at later stages *(3)*, a combination of subjective assessments throughout development may further improve the chances of selecting the single most viable blastocyst from the cohort and improve the implantation rate and the number of healthy offspring born as a result of assisted reproduction procedures.

Endometrial suitability for embryo transfer

Embryo implantation is the result of the successful interaction between the embryo and the endometrium *(272)*. Increasing evidence indicates that steroid-induced molecules acting as paracrine modulators are necessary for embryo–uterine interactions. Successful implantation, therefore, is determined both by the quality of the embryo and the receptiveness of the endometrium.

To further improve pregnancy rates, it is clear that ART would benefit substantially from being able to determine the exact timing of endometrial receptivity (the implantation window). It is generally believed that this window of opportunity occurs between Days 18 and 24 of the normal menstrual cycle *(273)*. However, this window may not be absolute and considerable interindividual variability may exist. Hence, the need to determine for each patient whether the endometrium is adequately prepared at the time of embryo transfer.

Currently available technology for the assessment of the endometrium prior to embryo transfer can be divided into microscopic assessment of endometrial biopsies and imaging techniques.

Microscopic assessment of endometrial biopsies

The major disadvantages of endometrial biopsies are their invasiveness, their negative impact on the integrity of the endometrium and their interference with the implantation process itself. These techniques should only be used in unstimulated cycles prior to ART. However, the extent to which assessments performed in a natural cycle are predictive of the quality of the endometrium in a subsequent stimulated cycle has not yet been studied. In addition, the literature is unclear about the intraindividual variation from cycle to cycle. Some uncertainty also exists as to whether one biopsy per cycle is sufficient for the assessment of receptivity.

Histological dating used in the assessment of morphological markers has shown that the timing of biopsy, the methods used for chronological standardization, and the extent of discrepancy required to define an endometrial biopsy as being “out of phase” remains unsettled *(274)*. A high inter- and intra-observer variation has further limited the clinical utility of traditional dating techniques.

The formation of pinopods, which are sponge-like smooth membrane projections that arise from the entire surface of endometrial cells lining the uterine cavity, has been related to the presumed time of blastocyst implantation *(275)*. They are detected by electron microscopy, making it a cumbersome and expensive technique for use in a clinical setting. Conventional light microscopy has been shown to be an unreliable technique for the detection of pinopods *(276)*.

There is a large body of evidence suggesting that numerous factors are involved in human implantation. The expanding group of potentially important factors includes mucins, integrins, trophinin/tastin, EGF, HB-EGF, amphiregulin, CSF-1, LIF, IL-1β, calcitonin, HOXa-

10 and COX-2. Most of these biomarkers are typically expressed around the time of the implantation window. However, most of these putative markers of uterine receptivity have no proven clinical relevance to date. The expression of β3 and α4 integrins has been studied extensively. These molecules are considered likely to correlate to uterine receptivity. While patterns of integrin expression cannot be used to accurately date the endometrium *(274)*, they may reveal subgroups of endometrial dysfunction in patients in the absence of morphological abnormalities identified at the light microscopic level *(277–279)*. Although this information may be useful when advising the patient about the cause of her infertility, there are no objective data to show that integrin expression patterns actually predict endometrial receptivity.

Imaging techniques (ultrasound and magnetic resonance imaging)

Because of their noninvasive nature, imaging techniques are ideally suited to assess the endometrium immediately prior to embryo transfer. In contrast to magnetic resonance imaging (MRI), ultrasonography is a much cheaper and more widely available imaging technique. However, the spatial resolution (degree of detail) of MRI is superior to that of ultrasonography.

Conflicting results have been reported regarding the correlation between the thickness of the endometrium and pregnancy rates following ART *(280–289)*. Measurement of endometrial thickness, or even endometrial volume, with three-dimensional ultrasound techniques does not yield better results *(290)*. This may be explained by the fact that the thickness of the endometrium has been shown to be more dependent on the time of exposure to estrogen rather than the dose of estrogen exposure *(288)*. In addition, important interindividual variation in endometrial thickness on the day of hCG administration has been noted.

The echogenicity of a tissue is a measure of its capability to reflect ultrasound waves. Some studies have shown that endometrial echogenicity in the late follicular phase predicts ART outcome *(280,283,288, 291–293)*. Others, however, have failed to find a relation between endometrial echogenicity and implantation rates *(294, 295)*. This controversy may be explained by operator-dependent variability, the use of arbitrary and heterogeneous classifications, and the lack of control for confounding factors (e.g. poor embryo quality and uterine cavity abnormalities) that influence the analysis of results. In an encouraging study, endometrial echogenicity on the day of hCG administration was assessed objectively with a computer-assisted module for the analysis of ultrasound images in a selected subset of ART patients *(289)*. In this study, echogenicity patterns were strongly correlated with implantation rates. However, the diagnostic value in an unselected population of patients remains to be determined.

Endometrial blood flow may be used as a functional marker and since the development of power Doppler sonography and, more recently, three-dimensional power Doppler sonography, it has become possible to evaluate the vessel density and perfusion in the endometrial and subendometrial layers in a quantitative way. Most studies so far have been rather small and conflicting results have frequently been reported *(296–301)*.

The junctional zone which is the myometrial layer just underlying the endometrium, has recently been described as a separate functional unit. One of the functional properties of this layer is that it produces contraction waves. Contractions can be directed towards the uterine fundus, the cervix, or they can be chaotic or opposing. These contractions have been demonstrated to be strong enough to displace embryos in the uterine cavity *(204)*. The direction and amplitude of these contraction waves are stimulated by hormonal influences *(302)* and physical stimuli (e.g. difficult transfer) *(204,206)*. One study was controlled for confounding variables and uterine contractions were assessed objectively by a computerized system *(229)*. In the selected patient population, high frequency contractions on the day of embryo transfer were found to decrease implantation and pregnancy rates. A negative correlation between uterine contraction frequency and serum progesterone concentrations was also observed, illustrating the uterine relaxant properties of progesterone.

Ultrasonography clearly has a number of major advantages (low cost, wide availability, possibilities for standardization using computer software), making it the area with the greatest potential for clinical application. However, to become generally accepted, any assessment will need to be rigorously tested for its diagnostic value. Although some tests have been shown to be quite promising in selected subpopulations of patients, these same tests need to be re-evaluated in unselected patient populations. In these

evaluations, investigators will need to report on commonly used parameters such as sensitivity, specificity, positive predictive value and negative predictive value. In particular, receiver–operator curves should be provided with each test.

A further area of research will have to focus on how the results of these endometrial assessments can assist the clinician in achieving better outcomes for the patient. Some authors have suggested that fresh embryo transfers may need to be delayed in the event of an unfavourable assessment of endometrial receptivity. It seems unlikely that many patients will accept this decision and therefore research efforts should concentrate on the development of medical interventions that may improve endometrial receptivity.

Luteal phase support

One area that has received a lot of attention is the need for luteal phase support in downregulated cycles. Since the original articles of Smitz *et al. (303)* and Wildt *et al. (304)* were published, the use of luteal support in downregulated ART cycles has been accepted as best medical practice. Various methods have been described to support the luteal phase. The use of hCG injections has been abandoned in most centres in favour of progesterone. The long half-life of hCG and its direct stimulation of the ovary contribute to the associated increased risk of ovarian hyperstimulation syndrome (OHSS) *(305)*. Progesterone can be administered in a variety of ways. The intramuscular and vaginal routes are currently the most widely adopted. Orally administered progesterone is rapidly metabolized in the gastrointestinal tract and its use has proved to be inferior *(306)*. A lot of controversy still exists as to whether the vaginal route results in better secretory endometrial transformation. This controversy stems from the fact that vaginally administered progesterone results in adequate secretory endometrial transformation, despite serum progesterone values lower than those observed after intramuscular administration, even if they are lower than those observed during the luteal phase of the natural cycle. This discrepancy is indicative of the first uterine-pass effect and therefore of a better bioavailability of progesterone in the uterus *(306)*.

Recent research has highlighted the detrimental effects of high estradiol levels on implantation *(307,308)*. Implantation rates are lower in patients who are high responders. The implantation and pregnancy rates were correlated to the peak estradiol concentration, regardless of the number of oocytes collected *(309)*. The effect of estradiol was mediated through endometrial receptivity as demonstrated in a study involving oocyte donors. The implantation rates in recipients of embryos derived from high responders were similar to normal responders *(309)*. It has been suggested that stimulation protocols aimed at reducing the follicular response may overcome the low implantation rates in high responders. Simon *et al. (309)* used the step-down protocol, originally proposed by Fauser *et al. (310)*, to successfully improve both the implantation and pregnancy rates in high responders. This involved the administration of 100 or 150 IU/day recombinant FSH starting on cycle Day 5 *(311)*. From cycle Day 8 or later, cotreatment was begun with 0.25 mg/day GnRH antagonist. No luteal support was provided. This pilot study demonstrated that IVF is feasible with a minimal stimulation approach and that luteal support may not be necessary.

This also raises the issue of whether the use of the short-acting gonadotrophin-releasing hormone (GnRH) antagonists is likely to change the need for luteal phase support. The pilot study of de Jong *et al. (311)* seems to suggest that luteal support is not necessary following GnRH antagonist administration in an IVF cycle. This finding is in direct contrast to the results from another small study that concluded that corpus luteum function may be impaired in cycles stimulated with hMG and a GnRH antagonist *(312)*.

Antiphospholipids

In a completely different area of research, mounting evidence suggests that inheritable thrombophilias, such as activated protein C resistance, Factor V Leiden mutation, or hyperhomocysteinaemia are associated with an increased risk of fetal loss and pre-eclampsia. Acquired thrombophilias, such as the antiphospholipid syndrome (APS), are also emerging as an important cause of recurrent pregnancy loss. The common pathogenic pathway is thought to be slow progressive thrombosis and infarction in the placenta. For patients with APS who have a history of thrombosis or recurrent pregnancy losses, heparin plus low-dose aspirin appears to be the regimen of choice *(313,314)*. Interestingly, antiphospholipid antibodies (APA) have also been shown to interact with syncytiotrophoblast and cytotrophoblast layers and could, therefore, theoretically affect implantation.

Several trials of treatment with heparin and aspirin in women with positive APA undergoing IVF have been completed. Although none of the studies were randomized, prospective, blinded trials there does not appear to be a significant effect of heparin–aspirin treatment on implantation rate, pregnancy rate, or ongoing pregnancy rate. Furthermore, it should be stressed that heparin–aspirin treatment may not be without complications. One maternal death has been reported associated with heparin–aspirin treatment in IVF, due to a cerebral haemorrhage in a nine-week pregnant woman carrying triplets *(315)*. Subcutaneous heparin does not cross the placenta and therefore has no adverse effects on the fetus, but potential side-effects for the mother include bleeding, thrombocytopenia and osteoporosis.

Antibiotics

Recently, there has been growing interest in the effect of infectious agents on ART pregnancy rates. The assumption is that women with specific vaginal pathogens may have an increased incidence of endometritis, which would lead to a reduced implantation rate. Although it is part of good clinical practice to treat any clinically manifest genital tract infection, it is unclear whether screening for microorganisms should be routine. One study assessed the impact of individual bacteria isolated from the vagina and the tip of the embryo transfer catheter on livebirth rates *(316)*. It was found that different types of bacteria recovered from the embryo transfer catheter had variable effects on live-birth rates. Prophylactic doxycycline had little effect on the vaginal flora.

The issue of whether bacterial vaginosis, if present at the time of oocyte recovery, adversely affects fertilization and implantation has been investigated more closely. The prevalence of bacterial vaginosis was much higher in infertile patients undergoing ART treatment than found by others in antenatal and general gynaecological populations *(317)*. In all studies to date, no significant effect of bacterial vaginosis on fertilization and implantation rates has been demonstrated *(317–319)*. Therefore, routine screening and treatment for bacterial vaginosis before ART treatment would appear to be unwarranted.

Furthermore, antibiotic therapy may increase the likelihood of inoculation of antibiotic-resistant pathogenic bacteria from the vagina into the embryo culture system during vaginal oocyte collection *(320)*. Whether screening and treatment of bacterial vaginosis would result in a reduction in later complications during pregnancy remains an open question *(317)*.

In summary, ultrasound techniques seem to hold the greatest promise of becoming clinically useful tools to assess endometrial receptivity. Most of the ultrasound techniques still await proper validation in unselected patient populations. Further research is required to investigate how patients with a poorly prepared endometrium should be managed clinically.

Impact on offspring

Since the birth of the first IVF baby in 1978, several hundred thousand babies have been born worldwide as a result of assisted conception. Several international registers of births resulting from IVF have been established to enable assessment of the health of these children and several analyses of the data recorded in these registers have been published to date and are summarized in Table 1.

The majority of studies have demonstrated no major differences in outcomes for singleton pregnancies except perhaps for an increased incidence of premature and low-birth-weight babies. However, these findings seem to be dependent more on patient characteristics than on the ART per se, as no major differences can be found for ART children compared to the general population when patients are matched for parity, maternal age and year of delivery *(321, 322)*.

By far the greatest adverse impact on offspring born as a result of ART is as a direct result of the increased incidence of multiple pregnancy. The

Table 1. Summary of national registers of ART and outcomes

Publication reference	Register	Years
(330,338,340,372–378)	Australia and New Zealand	1979–1997
(321)	Denmark	1994–1995
(332)	Finland	1991–1993
(328,329)	France	1986–1990
(327)	Great Britain	1978–1987
(337)	Israel	1982–1989
(323)	Sweden	1982–1985
(339,379–387)	USA and Canada	1988–1997

Swedish registry study showed a 20-fold increased risk of being born as a multiple birth baby for an ART child compared with the general population *(323)*. The last world collaborative report on ART recorded a multiple birth rate of 29%, the majority of which were twins *(324)*. Multiple birth infants, regardless of whether they originate from ART or spontaneous conception, have an increased risk of preterm delivery, low birth weight, congenital malformations, fetal and infant deaths and long-term morbidity and disability as survivors *(325,326)*. There is a fivefold increase in premature delivery of children resulting from ART *(323)*. This can be explained in the main part by the incidence of multiple births but is also true for singleton births *(323,327–331)*.

There is also an increased incidence of low-birth-weight babies following ART compared to the general population, even when only singleton pregnancies are considered*(323,327–330,332)*. More recently, with the increasing use of extended culture followed by blastocyst transfer, concerns have been expressed about the potential for producing babies with high birth weights similar to the "large offspring syndrome" reported following blastocyst transfer in domestic animal species *(333,334)*. However, human infants conceived following blastocyst transfer are not significantly different in birth weight from infants conceived spontaneously *(335)* or from infants conceived following transfer of early cleavage-stage embryos *(336)*. Perinatal and infant mortality is 1.7–3 times the national average *(323,327–330,337)* and, in some ART populations, is accounted for entirely by the high percentage of multiple births *(327,328)*.

Children born as a result of ART have an increased risk of malformations compared with the general population; however, this risk can be partly explained by the high proportion of multiple births in the ART group *(323)*. An increased incidence of neural tube defects (anencephaly, hydrocephaly, spina bifida) *(323,327,328,338)*, oesophageal atresia *(323)* and transposition of the great vessels *(338)* has been reported for ART infants. In the Swedish study, ten ART infants (0.2%) had neural tube defects (anencephaly and spina bifida) compared with the expected number of three to four. Seven of these ten infants and six of seven ART infants with hydrocephalus were from sets of twins *(323)*. Similarly, in the Australian study, three of six infants born with spina bifida and two of four infants born with transposition of the great vessels were from multiple births *(338)*. Other studies have reported no increase in congenital malformations in large numbers of ART children, despite a very high incidence of multiple pregnancy *(339)*.

Multiple pregnancies are also associated with increased infant and childhood morbidity such as cerebral palsy and mental retardation, due primarily to the increased incidence of prematurity and low birth weight *(325,326)*. The Swedish study failed to identify any increase in the incidence of cancer in children resulting from ART *(323)* which is in contrast to a previous report indicating a possible increase in neuroectodermal tumours *(340)*. Most follow-up studies of children born as a result of ART have not been running for long enough to assess the risks of long-term handicap.

A follow-up study of singleton infants conceived by ART to first-time mothers for the first year following birth has shown no difference in mental, motor, speech and social development compared to a matched control from the general population *(341)*. Similarly, the mental development of children conceived by ART at the age of 12 months was normal and not different from matched controls *(342)*. However, children of multiple births score lower on average, both on physical and mental scales *(342)*. Brandes and co-workers *(342)* concluded that when an ART pregnancy is carried to term, yielding an apparently healthy infant, the infant can be expected to develop and thrive similarly to non-ART-conceived peers. Similarly, Wennerholm and co-workers *(331)* concluded that if the neonatal period is uncomplicated, subsequent growth and development of children conceived by ART will be normal. In studies of older children, no independent ART effect has been found for growth and physical outcome (when matched for plurality and weeks of gestation) *(343)* or for cognitive, behavioural and social development*(344)*.

In very large studies, the sex ratio for births resulting from ART does not differ significantly from the national ratios *(327,328),* although it has been suggested that sex selection may be inadvertently performed in ART programmes by selecting for the faster cleaving embryos *(345)*. It has, however, been reported that the sex ratio for births resulting from blastocyst transfer, when the fastest cleaving embryos are selected for transfer, favours males and represents a significant shift in the sex ratio for births resulting from spontaneous conceptions *(335)*.

The high incidence of multiple births following assisted conception is largely due to the practice of transferring multiple embryos. This practice has arisen from the data indicating that at least for the first three

to four early cleavage-stage embryos transferred, there is a positive correlation between the number of embryos transferred and the pregnancy rate *(346)*. However, progress in the past decade has seen the introduction of improved culture media, culture conditions and selection criteria for embryo transfer and the corresponding implantation rates have increased dramatically. The improvement in implantation rates allows, for the first time, the consideration of reducing the number of embryos for transfer to two *(152,250,347–356)*, and possibly even one *(120, 149,151,357,358)*, to eliminate the risks associated with multiple pregnancy whilst still maintaining high pregnancy rates.

However, reducing the number of embryos for transfer will not entirely eliminate multiple pregnancies. Monozygotic twinning (identical twins formed from the one embryo) has been reported to be higher following assisted reproduction *(359,360)* than in the general population. Monozygous twins, and in particular monozygous, monochorionic twins (identical twins which share the same chorion), add significantly to the risks associated with multiple pregnancy and to the poorer health and survival of offspring. The incidence of monozygotic twinning has been reported to be very high in the subgroup of ART patients whose embryos have been zona manipulated, either for assisted fertilization (SUZI or ICSI) or assisted hatching (creating a small hole in the zona by mechanical, chemical or laser methods) *(361–364)*. Others, however, have reported that the frequency of monozygotic twinning is not different for patients whose embryos have been zona manipulated and those that remain zona intact *(365)*. The incidence of monozygotic twinning has also been reported to be very high following blastocyst transfer *(366,367)*.

Although the exact mechanism of monozygotic twin formation in ART is unknown, it has been ascribed to ovulation induction *(368)*, ART culture conditions *(359)*, zona architecture or micromanipulation *(364)*, or asynchrony between the uterus and embryo *(369)*. The observation that the incidence of monozygotic twinning following ovulation induction is also higher than that of the general population *(368)* suggests a role for hormonal manipulation rather than *in vitro* embryo culture conditions. Alterations in the hormonal milieu may lead to delays in oocyte or embryo transport and implantation at crucial developmental moments resulting in induction of twinning *(360)*. Alternatively, exposure to high concentrations of gonadotrophins may lead to zona hardening *(370)* resulting in impaired hatching at the blastocyst stage and retention of part of the embryo within the zona. This could result in two separate embryos forming if the inner cell mass is bisected *(371)*. Exposure to *in vitro* culture conditions rather than oviductal and uterine secretions containing lysins may similarly result in zona hardening and impaired hatching *(359)*. The higher incidence of monozygotic twinning observed following transfer of embryos that have undergone zona manipulation suggests an additional role for the architecture of the zona *(364)*. The dimensions of the artificial gap created during assisted fertilization or assisted hatching, particularly if small, may impose a physical restriction to the emerging embryo causing it to split *(364)*. The size of the artificial gap, however, varies significantly according to the method of assisted hatching and according to the degree of technical expertise of the embryologist performing the procedure. This variation may help to explain the discrepancy in the reported incidence of monozygotic twinning following assisted hatching *(365)*. Zona trapping, however, cannot be the entire explanation for the increased incidence of monozygotic twinning reported following blastocyst transfer, as the incidence remains high despite transfer of blastocysts that have had the zona chemically removed prior to transfer (G.M. Jones and A.O. Trounson, personal communication). The findings of Meintjes *et al.* *(369)* that the incidence of monozygotic twinning following blastocyst transfer and in patients using donor oocytes lends some support to the idea that monozygotic twinning arises due to some compromised endometrial–embryo communication and subsequent asynchrony between the uterus and endometrium.

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