Can the developmental competence of early human embryos be predicted effectively in the clinical IVF laboratory?

Jonathan Van Blerkom

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado, 80309 and Reproductive Genetics In Vitro, Denver, Colorado, 80222, USA

Experience with clinical in-vitro fertilization (IVF) over the last two decades has profoundly changed our understanding of the developmental biology of human oocytes and preimplantation stage embryos, and has provided important insights and practical lessons concerning the proper laboratory management of gametes and embryos. The progressive increase in successful outcomes from IVF reported by most programmes can be attributed to the introduction of techniques such as intracytoplasmic sperm injection (ICSI) and to greater technical experience in the IVF laboratory. While improved success rates in infertility treatment are a tribute to the efforts of those involved in the IVF enterprise, in the US, it clearly has come at the cost of an increased frequency of higher order gestations and a marked growth in the requirement for and utilization of selective fetal reduction (Faber, 1997). It is unclear to what extent the following factors, either individually or in combination, have contributed to this increase: (i) significant and beneficial changes in laboratory procedures, including embryo culture, (ii) the persistence of the notion that the more embryos replaced the more likely it is that pregnancy will result, or (iii) a qualitative change in the population of patients for which IVF and derivative procedures are truly effective. However, if human embryos with high developmental competence are now being produced in numbers greater than those routinely obtained only several years ago, then is the ‘state of the art’ sufficient to predict developmental potential prior to transfer such that the number of embryos replaced will not reduce pregnancy rates but will reduce the probability of multiple gestations?

To answer this question, it is necessary to address the issue of what proportion of embryos obtained by IVF can be expected to be developmentally viable after transfer. Earlier studies that examined outcomes based on large numbers of patients and embryos concluded that each embryo had a unique developmental potential, and that for any particular IVF cycle only a relatively small proportion of cleavage stage embryos were competent to implant and develop through gestation (Acosta et al., 1988; see review by Van Blerkom, 1994a). This conclusion was consistent with observations from fertile women attempting to conceive, and from IVF findings in which embryos judged morphologically normal had arrested development during the early cleavage stages with very few developing into expanded blastocysts. Upon close inspection, even those embryos that developed into blastocysts were often reported to be developmentally retarded with respect to cell numbers or allocation of cells between the inner cell mass and tropheoblast (Winston et al., 1991; reviewed by Van Blerkom, 1994a). Many investigators attributed the poor developmental performance of human embryos in vitro to conditions or media that were inadequate or inappropriate for the human embryo (see Bavister and Boatman, 1997). This was an attractive notion owing to the fact that media in common use for clinical IVF had been developed decades earlier for specific types of cell and tissue culture rather than for the maintenance of the early human embryo. However, pregnancies did occur with embryos fertilized and grown in ‘older’ media such as Ham’s F10, and for many experienced programmes, pregnancy rates in the 30% range were routinely reported from at least the mid 1980s. Clearly, some proportion of embryos could develop normally in media considered inadequate for the human.

Developmentally lethal defects occur in the human female gamete prior to insemination

Detailed analyses of cellular and genetic characteristics of human oocytes indicate that chromosomal and cytoplasmic defects occur at a relatively high frequency. Of mature oocytes, ~25% contain numerical chromosomal disorders (aneuploidies) and structural defects (fragmentation) that arise during meiotic metaphase and predispose the fertilized egg to developmental failure (see review by Van Blerkom, 1994b). For a subset of women, substantially higher frequencies of aneuploidy have been reported (Wall et al., 1996) and for some, chromosomal abnormalities may be a proximate cause of their infertility (Zenzes et al., 1992). Unlike the oocytes of model systems such as the mouse, human oocytes often exhibit a pleiomorphic cytoplasm that is especially evident when high numbers of gametes are retrieved for IVF. While some of these cytoplasmic phenotypes represent developmentally lethal defects in organ-elle distribution or cytoplasmic organization, others are benign with respect to the implantation potential of the embryo (see review by Van Blerkom, 1994b). Some types of cytoplasmic defects are associated with very high frequencies of chromosomal disorder suggesting that subtle degenerative changes at the cellular level may also influence chromosomal normality. Indeed, recent evidence for apoptotic alterations with attendant chromosomal damage indicates another class of lethal defects that can affect the developmental competence of mature mouse and human oocytes (Fujino et al., 1996). Very different levels of metabolism and ATP production have been detected in mature human oocytes from the same and different patients (Magnusson et al., 1986; Van Blerkom et al., 1995). Although very low levels of metabolism do not prevent meiotic maturation, cleavage stage embryos that result from such oocytes,
While normal in gross morphology, appear to have a very low developmental ability (Van Blerkom et al., 1995). Several investigators have reported that sperm penetration without cytoplasmic activation or male pronuclear evolution (silent fertilization) can occur in a relatively significant fraction (20–30%) of oocytes subjected to conventional IVF (Van Blerkom et al., 1994; Ash et al., 1995) or assisted insemination (Umer et al., 1993; Wall et al., 1996). This finding further demonstrates how the developmental heterogeneity of human oocytes can be expressed during the earliest stages of the fertilization process and, with the examples cited above, illustrates why no culture medium or system will rescue from developmental failure female gametes that at retrieval or ovulation are already compromised owing to inherent biochemical, cellular or genetic defects, many of which we are just beginning to recognize.

How embryos are currently assessed for developmental competence in most clinical IVF laboratories

The practice of grading embryos, with the assignment of a numerical or letter designation based on criteria such as degree of fragmentation and uniformity of blastomeres, has become standard in the IVF laboratory. While grading schemes have undergone modification over the years and may provide an ‘analytical’ aspect to an IVF cycle, the subjective or empirical basis of such morphological assessments and their ability to predict subsequent developmental competence has been repeatedly questioned in the literature. However, while all IVF programmes occasionally will observe normal pregnancies after the transfer of ‘poor’ grade embryos, most embryos that exhibit significant fragmentation or obvious abnormalities in cytotkinesis do not develop and indeed have been shown to be chromosomally abnormal (e.g. catastrophic mosaicisms: Munne et al., 1993). The presence of multinucleated blastomeres at the 2-cell stage is one of the more informative indicators of subsequent developmental failure owing to the extremely high frequency of numerical disorders in the chromosomal complement that affect both blastomeres in these embryos (Kligman et al., 1996). Nuclear membrane dissolution can occur in multinucleated blastomeres during the cell cycle and some of these embryos are capable of limited cleavage divisions. Consequently, the occurrence of these embryos could go unnoticed if inspections in the clinical laboratory are made only to confirm fertilization and to select embryos for replacement at the time of transfer. These findings indicate that morphological observations alone can be of some predictive value in assessing the relative developmental potential of oocytes and embryos, especially if evaluations are made during the 2-cell stage.

Recently, many IVF programmes have extended the duration of embryo culture by 24 or 48 h from the previous ‘standard’ of ~2 days. Embryos that fail to progress beyond the early cleavage stages are assumed to be developmentally non-viable and, depending upon the clinic’s protocol, may not be considered appropriate for transfer or cryopreservation. While this is an important and useful change for programmes that inspect embryos infrequently, it is my experience that most of the embryos which do not progress after 3 or 4 days of culture can be identified during the first 48–60 h. However, with the current aggressive protocols of ovarian stimulation used in clinical IVF, a typical cycle may have eight or more morphologically equivalent and apparently developmentally progressive embryos available for transfer on days 3 or 4. It is at the mid-latter stages of the preimplantation period that subjective morphological assessments of developmental normality become problematic for the following reasons: (i) at the late morula and expanding blastocyst stages, inspection by routine light microscopy is not an accurate approach to the determination of cell numbers, and (ii) even with optical sectioning and computer-assisted morphometry, it is often very difficult to distinguish between anuclear cytoplasts and anuclear cells in mitosis and to determine whether appropriate cell allocation between inner cell mass and trophoblast has occurred. The use of DNA specific fluorescent probes to estimate cell numbers by counting nuclei in living embryos is very effective for experimental purposes (Van Blerkom, 1993) but application to embryos destined for transfer is currently unacceptable. Findings from DNA fluorescent studies of human blastocysts demonstrate that actual cell numbers can differ significantly among embryos which appear equivalent at the light microscopic level (Hardy et al., 1989; Dokras et al., 1991; Van Blerkom, 1993). For many current IVF practitioners, attainment of the blastocyst stage is viewed as de-facto evidence that such embryos are developmentally normal.

Several recent studies suggest that certain co-culture systems (Feng et al., 1996; Vlad et al., 1996), modifications to existing media (Quinn et al., 1995) or simple removal of antibiotics (Magli et al., 1996) promote a pattern and rate of human embryo development that is closer to the in-vivo situation. Rarely, however, do studies of human embryo culture medium and conditions acknowledge the fact that the biochemical and cellular milieu the embryo experiences as it progresses through the Fallopian tube and uterus changes both spatially and temporally. In this respect, it is worthwhile to recall that the embryo does not travel through the reproductive tract in a fluid milieu comparable to the in-vitro condition, but rather is subjected to a complex biochemical and physically dynamic environment in which cleavage and blastocyst formation occur in very close proximity to very different cell types in the Fallopian tube and uterus respectively, that are themselves undergoing proliferation and differentiation. Because of focal and regional differences in biochemistry and cellular function and activity, what a human embryo actually sees, requires, or utilizes from its normal surroundings is largely unknown. However, successful development through the preimplantation stages in vitro in relatively simple medium and the fact that conventional IVF/embryo transfer bypasses the Fallopian tube entirely indicates that the biochemical requirements for early embryonic development may be rather minimal. Consequently, the detection of growth factors and cytokines in reproductive tissues may relate more to the activity of those cells than to embryotrophic influences that are absolutely required for normal development and therefore must be included in culture medium formulations. In attempts to design simple human embryo culture medium capable of supporting development from the 1-cell to the expanded blastocyst stages, Quinn et al.
(1995) and Gardener et al. (1996) have incorporated into their formulations metabolite and ionic concentrations derived from the analysis of metabolites and salts obtained from human reproductive tract flushes. Although the values obtained must be considered an approximation of what the embryo might actually experience in vivo, in-vitro results in which development to the expanded blastocyst stage is obtained at relatively high frequency tends to support the notion that the biochemical requirements for preimplantation human embryogenesis may not be complex.

Whether the ability of an embryo to progress to the blastocyst stage is a definitive indication of developmental potential is an issue of fundamental importance in the evolution of a standard culture medium and a set of laboratory protocols for clinical IVF. While attainment of this stage is clearly an important milestone in early embryogenesis, developmental normality cannot be assumed as both molecular and cellular studies have observed grossly normal appearing expanded blastocysts with developmentally significant molecular and cellular defects (Dokras et al., 1991, 1993; Van Blerkom, 1993; Turner and Lenton, 1996). For example, Turner and Lenton (1996) compared results obtained from human embryos cultured under conventional conditions to those cultured in the presence of Vero cells. Similar to many other findings from co-culture studies (see Van Blerkom, 1993), these investigators found that a higher number of embryos reached the blastocyst stage in the presence of Vero cells. However, their results showed for the co-cultured embryos no significant improvement in the morphology of the resultant blastocysts and the occurrence of a greater number of blastocysts that seemed to be functionally incompetent, as assessed by human chorionic gonadotrophin (HCG) production. For assessments of embryonic development at the blastocyst stage to be effective in the clinical IVF laboratory, not only should efforts be made to determine total cell numbers but, more critical to outcome, whether the inner cell mass is present and normal. The importance of such determinations was demonstrated by the findings of Winston et al. (1991), who reported that day 5 human blastocysts often exhibited an absent or deficient inner cell mass such that if implantation occurred, the resulting embryo would be expected either not to progress or to develop as an anembryonic pregnancy (so-called blighted ovum).

If new culture systems or media formulations are shown to be unambiguously capable of supporting normal human embryogenesis to the expanded blastocyst stage, then maintenance in vitro for 5 days becomes a very practical approach to embryo selection on the basis of demonstrated developmental potential. An additional benefit would be a reduction in the risk of higher order gestations without compromising the probability of pregnancy. However, the clinician will be faced with the difficult task of explaining to a subset of patients why no transfer will occur owing to the failure of the embryos to develop progressively. It is in this unfortunate situation (but not uncommon even when embryos are cultured for 3–3.5 days) that difficulty is experienced in explaining to a patient how an in-vitro system can be so predictive of developmental competence that it is equivalent to her uterus in the support of her embryos. In order not to disappoint patients, many programmes will replace embryos deemed non-progressive. Clearly, confidence in any assessment system is called into question when implantation occurs with such embryos. In this respect, while new media formulations and culture conditions directed specifically to the human embryo are essential in clinical IVF, it is equally important to identify and understand the origins of those defects in human oocytes that have developmental consequences for the embryo and which cannot be rescued or corrected by an optimized in-vitro environment.

New approaches to non-invasive assessments of human oocyte and embryo developmental potential

The ability to make reasonable predictions of oocyte developmental competence requires that the methodology used be non-invasive and applicable in a clinical IVF laboratory. To be of value in the selection of oocytes for insemination or embryos for transfer, a clear understanding of the biological processes that may be defective is essential. Experimental approaches to this issue have examined whether intrafollicular influences to which the oocyte may be exposed are associated with outcome after IVF. Quantitative analysis of follicular fluid steroids, proteins, enzymes, polypeptides, and growth factors have demonstrated the extraordinary complexity of follicular fluid but have not provided a definitive insight into what constitutes a normal intrafollicular environment or one that is clearly associated with high developmental competence. The behaviour of cumulus cells with respect to three specific oocyte/embryo–autonomous patterns of attachment, proliferation, and expansion during the first 24–48h of culture has been suggested to be predictive of the implantation potential of the corresponding embryo, which is cultured separately from the cumulus cells (Gregory et al., 1994). However, while the actual relevance of these phenotypes to IVF outcome remains to be determined unambiguously, they do suggest that basic cell biological processes of cumulus cells may be differentially influenced by follicle-specific conditions. If a developmentally significant relationship does indeed exist, it can provide an experimental approach to understanding the molecular basis of developmentally critical interactions between the oocyte and its supporting cells (Van Blerkom, 1996), and how differentiative events in the oocyte can be influenced by specific factors within the follicle. Although promising leads exist, no single follicular factor(s) that can be readily determined or measured in the IVF laboratory has been shown to provide the ‘magic bullet’ for a definitive prediction of developmental competence of the oocyte or the embryo. We have addressed this issue by asking whether differences in follicular fluid biochemistry, cumulus cell behaviour in vitro, and oocyte/embryo developmental competence are related consequences of a follicle-specific physiology that can be assessed non-invasively prior to or at ovum retrieval (Van Blerkom, 1996). Our findings from the analysis of >1000 follicles indicate that the percentage dissolved oxygen in follicular fluid measured at the time of ovum retrieval is associated with the developmental normality of the oocyte and with differences in follicular biochemistry (Van Blerkom et al., 1997). The percent dissolved oxygen in follicular fluids of similar volumes and aspirated
from follicles of the same size (18–21 mm) has ranged from <1 to ~5.5%. While oxygen content appears to be unrelated to the frequency of meiotic maturation, fertilization, and cleavage, most oocytes with cytoplasmic defects and high frequencies of chromosomal and spindle disorders originate from severely hypoxic follicles, as do cleavage stage embryos with multinucleated blastomeres. Preliminary findings indicate that oocytes from follicles with oxygen contents below ~1.5% have low ATP contents (Van Blerkom et al., 1995) and an acidic intracellular pH (Van Blerkom, 1996) that may be associated with abnormalities in spindles and microtubules (Van Blerkom et al., 1996). Both retrospective and ongoing prospective findings strongly suggest that embryos derived from follicles with oxygen contents at or above ~3% are more likely to implant than if derived from severely hypoxic follicles.

At present, the dissolved oxygen content of follicular fluid cannot be predicted by any follicular characteristic observable during routine ultrasonographic examination. However, the addition of colour pulsed Doppler ultrasonography provides quantitative values associated with perifollicular blood flow and microvasculature development that do correlate with the oxygen content of follicular fluid measured at aspiration (Van Blerkom et al., 1997). To date, colour pulsed Doppler analysis of several hundred follicles has demonstrated that the degree of perifollicular vasculization is follicle-specific such that adjacent follicles with virtually identical characteristics on routine ultrasonographic examination exhibit very different blood flow rates and degrees of perifollicular vascular development, both of which correlate with the percentage dissolved oxygen in the corresponding follicular fluid. Our findings demonstrate that for most IVF patients in which the same protocol of follicular stimulation was used and comparable numbers of follicles were aspirated, 30–40% of the follicles present at ovum retrieval had perifollicular blood flow patterns consistent with a level of follicular oxygenation >3%. Other follicles in these cohorts displayed little or no detectable perifollicular blood flow and dissolved oxygen contents in follicular fluid at or <2%.

An association between follicular oxygen content and embryo developmental competence is suggested by our current experience in which embryos are selected for transfer at 52–60 h post-insemination (6–10-cell stage) on the basis of morphological assessments of the oocyte (Van Blerkom and Henry, 1992) and embryo (made at 10–14 h intervals after insemination), and derivation from a follicle whose perifollicular blood flow values were consistent with an intrafollicular dissolved oxygen content of at least 3%. With no change from previous years in culture system or number of embryos replaced (Van Blerkom et al., 1997), the addition of colour pulsed Doppler findings to embryo selection has been associated with a significant increase in the rate of ongoing pregnancies, albeit with multiple gestations occurring at a higher frequency (>50%) than during any previous period (Faber, 1997). Very similar results have been reported recently by Nargund et al. (1996) and Chui et al. (1997) who observed that human embryos derived from follicles with high perifollicular blood flow rates showed a corresponding increase in implantation potential.

These preliminary findings suggest that the application of perifollicular blood flow characteristics to embryo selection may be of significant value in the identification of embryos with high implantation/developmental potential. However, this approach requires the ability to associate each oocyte and its corresponding follicle, a task that is often cumbersome and extremely difficult to accomplish when large numbers of follicles have been stimulated to grow. The brief application of colour pulsed Doppler to each follicle immediately preceding aspiration and the pooling of oocytes based on the degree of perifollicular vasculization can simplify the logistics of oocyte selection using this criterion. The relationship between vascularity and oxygen content we have observed may provide a physiological basis for the often observed differences in follicular biochemistry and cumulus cell function. Indeed, the degree of development of the perifollicular vasculature that appears to determine the level of intrafollicular oxygenation may itself be dependent upon the capacity of each follicle to produce and secrete angiogenic promoters such as vascular endothelial growth factor (Kumat et al., 1995; Van Blerkom et al., 1997). Continued analyses of follicular vascularity, oxygen content, and outcome will be required to determine whether this is just another ‘promising lead’ or one that actually provides applicable determinants of developmental competence. However, it is this type of approach that will ultimately provide critical insights into how physiological, biochemical and cellular factors interact within the follicle to produce an identifiable set of conditions that is consistent with developmental normality for the oocyte and high implantation potential for the resulting embryo. In this respect, our understanding of the complex and stage-specific nature of human follicular biochemistry, physiology, and cell biology has entered a new era of discovery in which the developing follicle is not just a source of steroid hormones, but a site of synthesis of growth factors, cytokines, and novel polypeptides whose precise function(s) in the reproductive process remains to be determined. The insights gained from these studies will not only provide fundamental knowledge related to the origins of human oocyte developmental heterogeneity, but identifiable factors that can assist in the diagnosis and treatment of infertility.

Acknowledgement
Studies of follicular fluid oxygenation and vascularity were supported by a grant from the National Institutes of Health (HD-31907).

References


Kligman, L., Benadiva, C., Alikani, M. and Munne, S. (1996) The presence of competition among IVF clinics with similar levels of preganancy rates that has occurred in most programmes reflects the additive effects of improvements in the clinical management of patients and in laboratory methods. With increased success has come the now commonplace occurrence of higher order gestations and the ‘epidemic’ of multifetal reductions. In a few short years we have radically changed the incidence of multiple gestation, yet we have virtually no idea of the long-term impact these changes will have on our patients, their children or society. There is ample evidence of the immediate costs of treating these complicated pregnancies and an entire practice, almost a growth industry itself, of multifetal reduction has been born as a result of the practice patterns of infertility specialists in the US. Clearly, the progressive increase in pregnancy rates that has occurred in most programmes reflects the additive effects of improvements in the clinical management of patients and in laboratory methods. With increased success has come the now commonplace occurrence of higher order gestations and the ‘epidemic’ of multifetal reductions. I believe that the roots of this phenomenon lie not in the inability among in-vitro fertilization (IVF) practitioners to recognize this dramatic change in outcome, but rather in the nature of competition among IVF clinics with similar levels of competence, and the intentions of those providing these services to attract new infertility patients or maintain those already in their programme. It is my belief that such pregnancies represent a failure rather than a success of the IVF enterprise. Selective intrauterine abortion is only the most obvious symptom of the larger issue of multiple gestation. I believe the rightness or wrongness of this practice has nothing to do with the abortion issue. Regardless of the pro-life feelings of a patient, she cannot ignore the poor outcome of higher order

K.Faber

In the practice of advanced reproductive medicine we have progressed from the miracle baby to the day of the litter. In a few short years we have radically changed the incidence of multiple gestation, yet we have virtually no idea of the long-term impact these changes will have on our patients, children or society. There is ample evidence of the immediate costs of treating these complicated pregnancies and an entire practice, almost a growth industry itself, of multifetal reduction has been born as a result of the practice patterns of infertility specialists in the US. Clearly, the progressive increase in pregnancy rates that has occurred in most programmes reflects the additive effects of improvements in the clinical management of patients and in laboratory methods. With increased success has come the now commonplace occurrence of higher order gestations and the ‘epidemic’ of multifetal reductions. I believe that the roots of this phenomenon lie not in the inability among in-vitro fertilization (IVF) practitioners to recognize this dramatic change in outcome, but rather in the nature of competition among IVF clinics with similar levels of competence, and the intentions of those providing these services to attract new infertility patients or maintain those already in their programme. It is my belief that such pregnancies represent a failure rather than a success of the IVF enterprise. Selective intrauterine abortion is only the most obvious symptom of the larger issue of multiple gestation. I believe the rightness or wrongness of this practice has nothing to do with the abortion issue. Regardless of the pro-life feelings of a patient, she cannot ignore the poor outcome of higher order