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WHY SHOULD WE ASSESS OOCYTE AND EMBRYO MORPHOLOGY?

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INTRODUCTION

The ultimate goal of *in vitro* fertilization (IVF) is the establishment of a viable pregnancy and the birth of a single healthy baby. In order to meet the pressures imposed by anxious patients and by individual centers to improve success rates, multiple embryos are transferred in the hopes that one will implant. This practice has led to an increase in the incidence of higher order multiple pregnancies that present numerous complications to both the child and mother.

Multiple gestations have been accepted as a natural consequence of IVF. In fact, the incidence of twins is considered to be an extra bonus by both patients and clinicians. Since twin pregnancies can also lead to severe obstetric and neonatal complications, more and more infertility programs are striving to optimize stimulation regimens, laboratory and culture conditions, transfer procedures and post-transfer endocrine support in order to increase implantation rates and significantly reduce the number of embryos transferred. Several centers have already begun to establish a policy of elective transfer of a single embryo in good prognosis or “twin-prone” patients (ESHRE Campus Report, 2001). This policy, however, poses the somewhat daunting challenge of selecting and transferring one embryo without compromising pregnancy rates.

Historically, the most reliable method of embryo selection has been morphology, generally just prior to transfer. Early observations revealed embryos that appeared to look and develop “normally” were more likely to result in clinical pregnancies (Edwards *et al.*, 1984; Cummins *et al.*, 1986). This paper will review those characteristics reported to contribute to the definition the “golden” embryo – the one most likely to result in a pregnancy.

MORPHOLOGY ASSESSMENT: HOW AND WHEN?

The method of assessment of embryo or oocyte quality should not be detrimental to subsequent development. Selection methods should be not invasive or time-consuming, but still be predictive.

Embryo selection is not a static description at one point in time. Embryo morphology is based on a series of dynamic processes and influences that can change dramatically from moment to moment. Because of this, some researchers believe that embryo assessment should begin with the ovary and follicle (Van Blerkom, 1998, 2000; Bhal *et al.*, 1999; Van Blerkom *et al.*, 1997, 2001). Others maintain that the secret lies with the oocyte or pronuclear stage embryo (Ebner *et al.*, 1999, 2000; Manor *et al.*, 1999). This discussion will address several stages of embryo development, from the microenvironment of the follicle and the morphology of the oocyte to the pronuclear-, cleavage- and blastocyst-stage embryo (Rijnders and Jansen, 1998; Van Royen *et al.*, 1999; Gardner *et al.*, 2000). Efforts are constantly being made to further describe the

morphological characteristic of oocytes and embryos that will predict enhanced pregnancy and implantation rates.

MORPHOLOGY ASSESSMENT

The Follicle

Adequate vascularization has been associated with good follicle health in several species. In large domestic animals, such as the horse, ovulatory follicles demonstrate a dramatic increase in thecal blood flow, which has been correlated with high intrafollicular and circulating levels of estradiol, highly metabolic follicle cells and viable and healthy oocytes (Tucker *et al.*, 1991). Recently, it has been noted that perifollicular blood flow, as assessed by power color Doppler ultrasound, may not only be a good predictor of follicle health, but also of oocyte competence (Van Blerkom *et al.*, 1997; Huey *et al.*, 1999; Borini *et al.*, 2001). It has also been shown that follicular hypoxia may negatively affect spindle organization and mitochondrial and chromosomal segregation in the human oocyte, leading to abnormalities in the embryos that may result from it (Van Blerkom, 2000; Wilding *et al.* 2001). Good oocyte quality has also reported to be directly related to an increase in intrafollicular oxygenation. Other investigators have further demonstrated that follicular blood flow and the content of dissolved oxygen was positively related to the ability of the oocyte-embryo to develop successfully, compared with these from avascular follicles which did not result in any pregnancies (Chui *et al.*, 1997). Similar results were reported by Bhal and others (1999). These investigators found that significantly more pregnancies occurred when embryos from highly vascularized follicles were transferred (34.7%) compared to those from follicles with a low Doppler vascularity score (18%). Interestingly, no obvious differences in Day 3 embryo morphology were observed between the two groups. Huey and co-workers (1999) also found a significant correlation between oxygen content and oocyte-embryo health, but were not able to correlate this with differences in Doppler patterns. This discrepancy with other published reports may possibly be due to differences in equipment or technical methods.

The Oocyte

Ovarian stimulation protocols make numerous oocytes available per cycle and it is has been shown that a large variability exists within a single cohort (Van Blerkom, 2000). Several studies have been performed to precisely define the morphology of these oocytes and correlate that with a higher incidence of fertilization, embryo development and pregnancy rates. A large and detailed analysis of over 400 IVF cycles (>4,000 oocytes) demonstrated that pregnancy rates increased when more oocytes were retrieved within an individual IVF cycle. Although the presence of fractured zonae resulted in lower fertilization rates, no morphological characteristic was associated with pregnancy rates (Wittermer *et al.*, 2000).

Others have reported that better fertilization and embryo development rates occurred with "good quality" oocytes; those with a small perivitelline space and a regular and distinct first polar body (PB) (Suppinyopong *et al.* 2000). Additional reports have indicated that the morphology of the first PB can be a reliable indicator of oocyte age and that the presence of a well-shaped, non-fragmented PB was associated with increased pregnancy rates (Ebner *et al.*, 1999).

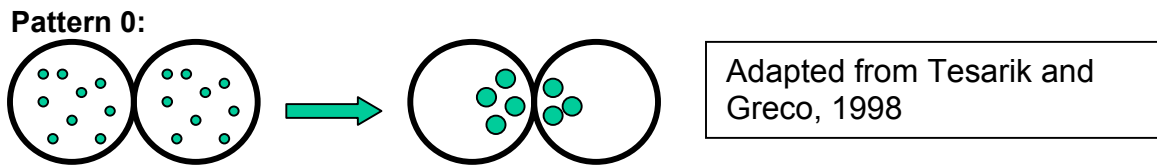
Why is PB morphology an important indicator of oocyte health? Battaglia and co-workers (1996) maintained that an irregular PB may be indicative of abnormalities associated with the mitotic spindle. The spindle, composed of microtubules, is necessary for normal alignment and separation of the maternal chromosomes during Meiosis I and II. As with the PB, healthy, intact spindles were found most often in oocytes from young women. Disruption or absence of the spindle has been shown to result in aneuploid embryos (Wang *et al.*, 2001). These investigators also observed that the presence of a birefringent spindle, viewed with a polarized microscope (Polscope®) was highly predictive of better fertilization and pregnancy rates.

The cytoplasm of the oocyte is also thought to be predictive of treatment success in IVF. Kahraman and others (2000) believed that an increase in cytoplasmic granularity was associated with low success rates after intracytoplasmic sperm injection (ICSI). They reported that centrally located cytoplasmic granulation (CLCG) in oocytes was associated with a lower pregnancy rate, due primarily to an extremely high abortion rate (54.5%) and therefore, urged that patients with a high incidence of CLCG be counseled with regard to a possible negative outcome.

The Pronuclear Embryo (Zygote)

Some investigators agree that the pronuclear (PN) stage of embryo development can be a very powerful predictor of subsequent embryo development, blastulation rate, the incidence of genetic abnormalities and resulting pregnancy rates (Tesarik and Greco, 1998; Manor *et al.*, 1999; Gerris and Van Royen, 2000; Scott *et al.*, 2000; Wittemer *et al.*, 2000; Balaban *et al.*, 2001). The shape, position and number of PNs in the embryo have been correlated with embryo health and overall success. Normally appearing zygotes contain two distinct PNs, but it is not unusual to see zygotes with a single pronucleus. Although a fairly large percentage of these embryos turned out to be diploid, based on fluorescent in situ hybridization (FISH), they still were not always karyotypically normal. Interestingly, even seemingly normal, bi-pronuclear embryos displayed abnormal or delayed cleavage, primarily in older women, and despite being diploid, were often chaotic in their chromosomal constitution (Lim *et al.*, 2000). The relative size of the PNs has also been shown to be informative. Manor and co-workers (1999) reported that chromosome abnormalities were present in 88.5% of ICSI and in 50% of IVF zygotes exhibiting unequally sized PNs.

Other studies have shown that PN size is not the only predictive parameter readily observed in the zygote. Several groups have concluded that the pattern of nucleolar precursor bodies (NPB) distribution within the PN may be the most reliable characteristic in determining embryo quality and pregnancy rate (Tesarik and Greco, 1998; Scott and Smith, 1998; Gerris and Van Royen, 2000; Balaban *et al.*, 2001; Scott *et al.*, 2000). Several patterns of NPB distribution have been documented and, based on this feature, Tesarik and Greco (1998) devised an initial classification system. Zygotes were divided into 6 different categories, one normal (Pattern 0) and 5 abnormal (Patterns 1-5). Pattern 0 zygotes had two PNs at the same stage of development; that is, NPB should be polarized at the time of evaluation. Several researchers in this field agree that Pattern 0 (see below) is the most predictive and has been reported to lead to a 72% blastocyst rate (Scott and Smith, 1998; Gerris and Van Royen, 2000; Balaban *et al.*, 2001). Higher implantation and pregnancy rates were obtained when at least 1 blastocyst derived from a 0 Pattern zygote was transferred. No relationship has been observed between Pattern 0 and female age or cause of infertility (Wittemer *et al.*, 2000).



The formation of the PNs and extrusion of the second PB have been extensively documented using time-lapse video. Payne and co-workers (1997) have described the appearance of periodic waves of granulation in the ooplasm. This “halo” of granulation is in contrast with the static granulation previously reported to occur in sub-optimal oocytes. Scott and co-workers (2000) have suggested that good quality embryos result from PN embryos exhibiting a peripheral “halo” and centrally located granulation and in an earlier study by this group, impressive pregnancy rates were achieved when 2 zygotes were transferred that displayed both the “halo” and polarized PNs (55%). In contrast, a study by Hurst and others (1998) demonstrated that selection of “good” zygotes for a Day 1 transfer resulted in severely reduced pregnancy rates when compared Day 2 transfer results (15% vs. 67%, respectively). A later study also found that, although cleavage-stage morphology was better in embryos derived from “good” quality zygotes (“halo” and polarized PNs), no differences in pregnancy rates were seen when compared to those from sub-standard zygotes (one or both PNs with scattered NPB) (Salumets *et al.*, 2001). In all cases, the best cleavage-stage embryos were transferred.

The Cleaving /Compacting Embryo

Most embryologists and clinicians are well aware of the general morphological characteristics that have been correlated with a good quality embryo and excellent atlases are available that describe and beautifully illustrate embryos of all stages and qualities (Veeck *et al.*, 1999; Menezo *et al.*, 1999). In view of this, a detailed description of embryo quality will not be included in this review. In general, “good” quality cleaving embryos display stage-specific cell division, have blastomeres of fairly equal size with few to no cytoplasmic fragments. Recent studies continue to add new information to what has been reported regarding the appearance of a good quality embryo and the processes it undergoes before implantation.

The phenomenon of uneven blastomere cleavage and how it relates to embryos health is not fully understood. Hardarson and co-workers (2001) demonstrated that embryos with unequal cell division had a lower developmental capacity which was attributed to the higher degree of genetic abnormalities detected by fluorescent in situ hybridization (FISH) (i.e. aneuploidy or multinucleated blastomeres). When otherwise good quality embryos were transferred, these investigators found that implantation and pregnancy rates were significantly lower when those with uneven-sized blastomeres were replaced [IR: 23.9 (uneven) vs. 36.4% (even); PR: 29.4 (uneven) vs. 36.4% (even)].

A fairly recent study by Desai and co-workers (2000) observed that cytoplasmic “pitting” occurs in some embryos, which thought to be physically different from excessive granulation in the cytoplasm. These researchers further ascertained that a high degree of pitting in Day 3 embryos could be used as an additional morphological marker for poor prognosis embryos.

A more familiar indication of embryo health and developmental potential is the degree of fragmentation. The actual cause(s) and mechanisms of fragmentation are still not well known. It has been proposed that generation of high levels of reactive oxygen

species (ROS) throughout the culture procedure contributes to excess fragment formation (Yang *et al.*, 1998). The presence of some fragmentation is not always indicative of poor embryo health and has been observed in embryos produced *in vivo* in a number of species (Alikani, 2000). Most groups will agree, however, that a high degree of fragmentation significantly reduces an embryo's implantation rate and may be associated with an elevated percentage of apoptotic features and chromosomal disorders (Ebner *et al.*, 2001). Patients should, therefore, be advised of any potential risks if only highly fragmented embryos are available for transfer.

Additional research in this area of embryo quality assessment has demonstrated that several types and patterns of fragmentation exist, leading to different IVF outcomes. Specifically, five different types of fragmentation have been described (Alikani, review; 2000). Types I and II are associated with the lysis of an individual blastomere. Type III, where fragments are scattered throughout the embryo, is the most common. Types I, II and III are usually seen in embryos from younger women (<38 years). Types IV and V describe embryos with more than 50% fragmentation (based on total volume) and are the patterns least likely to result in a pregnancy, primarily because the remaining blastomeres are grainy and necrotic.

Some investigators have suggested that removal of fragments may offer some hope for highly fragmented embryos. Clinical observations suggest that removal of fragments may not only improve the cosmetic appearance of the embryo, but may, in fact, enhance subsequent cell division and compaction, and may contribute to increasing implantation rates for these embryos (Alikani, 2000). Fragmentation removal, however, is a very invasive procedure and any benefits derived from it are highly controversial.

The presence of fragments is not an all-or-nothing condition. Van Blerkom and co-workers (2001) performed an extensive time-lapse investigation of fragment dynamics in human embryos. Based on his observations, he concluded that not all forms of spontaneous fragmentation were necessarily lethal. In certain cases there appeared more "transient" fragments that disappeared either by re-absorption or lysis.

Even when considering all that we know about cleavage stage embryos morphology, it is still difficult to identify those embryos that will continue to develop normally, even in culture. Rijnders and Jansen (1998) demonstrated that even good quality embryos on Day 3 do not always develop into blastocysts on Day 5 (Grade 1 – 50% blastocyst rate) and that a surprisingly high number of lesser grade embryos do (Grade 3 – 25%). Other studies have reported similar results, with only 48% of the embryos chosen for transfer on Day 3 becoming blastocysts (Graham *et al.*, 2000).

The morula and compaction stage embryo may be considered to be more of a transitional structure and more difficult to evaluate. Pregnancies have occurred when morulae were transferred on Day 5 (Rijnders and Jansen, 1998), but to a much lesser extent than when stage-specific embryos (blastocysts) were transferred. It is unclear whether this is due to entirely to developmental stage or in part to a reduced ability to select the appropriate embryo for transfer. Good quality morulae are uniform in appearance (individual cells are difficult to distinguish) and early cavitation on Day 4 is a good predictor for normal blastocyst development. The formation of cell-cell junctions on Day 3 can also be considered a good predictor for blastocyst development *in vitro* and subsequent pregnancies (Papadakis and Tucker, 2001, personal observation).

In addition to cell number, degree of fragmentation, blastomere size and cytoplasmic pitting, one of the most diagnostic characteristics of the health and developmental potential of an individual embryo is the number of nuclei per blastomere (Pelinck *et al.*, 1998; Van Royen *et al.*, 1999; Gerris and Van Royen, 2000). Several investigators believe that the multinuclear blastomere (MNB) index is highly indicative of

embryos that will NOT lead to a pregnancy. Studies in Belgium have reported that embryos resulting from ICSI without MNBs that were chosen for transfer on Day 3 had a higher chance of implanting and leading to a viable pregnancy compared with control transfers that included at least one embryo with MNB (11.3 vs. 6.0% and 28.7 vs. 16.9%, respectively; Pelinck *et al.*, 1998). In 1999, Van Royen and others reported that the presence or absence of MNB in embryos from high-responder patients may be THE pivotal characteristic in determining the implantation potential of an otherwise good quality embryo. When two “top” embryos (no MNB) were transferred, pregnancy and implantation rates were 63% and 49%, respectively, with 57% incidence of twins. Conversely, the transfer of two non-top embryos (good quality Day 3 embryos, both with MNB) resulted in significantly lower success rates (PR – 23%, IR – 12%). When a mixed group of embryos were transferred (1 top + 1 non-top) results were more intermediate (PR – 58%, 21% twins; IR – 35%), suggesting that better outcomes depend on at least one embryo being transferred without any MNBs (Van Royen *et al.*, 1999; Van Royen and Gerris, 2000). These authors went so far as to recommend that embryos with MNB NOT be transferred.

The Blastocyst

The transfer of as few as two blastocysts has been shown, on average, to be effective in reducing the number of multiple gestations in patients with good ovarian response (Toledo *et al.*, 2000; Gardner *et al.*, 2000; Langley *et al.*, 2001) and capable of establishing pregnancies in women with previous IVF failures (Rijnders and Jansen, 1998). Generally, a good quality blastocyst contains a well-expanded blastocoelic cavity, homogenous trophoblast with multiple cell-cell contacts and distinct nuclei, and an inner cell mass that is clearly visible and intact. A large number of studies exist in the literature attesting to excellent success rates following blastocyst transfer, with implantation rates of 67% (Langley *et al.*, 2001). Gardner and co-workers (2000) also concluded that if at least one high scoring, good quality blastocyst is available for transfer, pregnancy rates as high as 60% overall could be achieved.

Selection of the “golden” blastocyst is also not well understood. Some investigators strongly believe that extending the culture of embryos to blastocysts represents a type of “natural selection” of those embryos that will be genetically normal and viable *in utero*. Despite all our current advances, there is still very little “natural” about the procedures involved in IVF and embryo culture. Studies have shown that blastocyst morphology alone cannot rule out the incidence of chromosomal abnormalities (mosaicisms) in these embryos (Eriskov and Verlinky, 1998) and that even trisomic embryos become blastocysts at a very respectable rate (37%) (Sandalinas *et al.*, 2001).

MORPHOLOGY ASSESSMENT: SUMMARY AND CONCLUSIONS

Oocyte maturation, fertilization and embryo development are complex and dynamic events and it is difficult to pin-point a single moment in development that will identify the embryo destined for implantation. We have seen that unique characteristics are present at virtually every stage of development that may be predictive of an embryo’s implantation potential. Even the rates of cell division and differentiation themselves can influence embryo selection. It is important to remember, therefore, that morphology assessment cannot be accomplished by a one-time observation. The visible features that help us select the “golden” oocyte or embryo may appear gradually

occur over time, starting with increased follicular size and vascularity, continuing with a healthy, mature oocyte that fertilizes normally, an embryo that divides regularly and in a timely fashion and has blastomeres that are homogeneous intact and have one distinct nucleus. Depending on the day of transfer, the chance of success can be further influenced with selection of the most competent blastocyst and an uneventful and atraumatic embryo transfer.

The “artificial” selection IVF programs exert is necessary for minimizing multiple gestations without compromising pregnancy rate and outcome. We are already faced with the reality of single embryo transfer for many of our patients; certainly those more likely to develop twin or triplet pregnancies. Selection of that “golden” embryo relies on the additive influence of distinct, yet related variables that will hopefully add up to the birth of a single healthy baby.

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