—Mini Review—

Evaluation of Embryo Quality by Metabolomics: A New Strategy to Aid Single Embryo Transfer

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Abstract: *One of the major dilemmas surrounding assisted reproductive technologies (ART) is the inability to precisely estimate the reproductive potential of individual embryos. A number of new assessment techniques are currently being developed to address this dilemma, including techniques using genomic, proteomic and metabolomic approaches. Here we describe a metabolomic approach to embryo assessment that has the ability to distinguish specific biomarkers in embryo culture media. This technology assesses modifications of the chemical composition of the embryo's surrounding medium using a rapid Near Infra Red (NIR) spectroscopy approach and generates a value termed the "viability or Via Test-E™ score". We also report relationships between the algorithm generated Via Test-E™ score and morphology assessment on individual day 2 embryos selected for single embryo transfer and the implantation rates when a cut off score for the viability score is used in relation to implantation. The added armoury of metabolomic profiling by NIR to current morphological assessment techniques might allow a greater discrimination for selection of embryos for transfer and has the potential to improve IVF outcomes. Currently, additional studies are being performed to investigate the true value of metabolomic profiling by NIR spectroscopy.*

Key words: *Metabolomics, In Vitro Fertilization, Embryo selection, Embryo metabolism, Spectroscopy*

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Introduction

Utilization of assisted reproductive technologies (ART) continues to increase annually worldwide. This trend is driven by the steady improvement in ART delivery rates, improving access to care in many areas, and the relative ineffectiveness of other treatment options. At the current time, more than one percent of all children born in the United States and Europe are from ART related conceptions.

The high success rates established through IVF are attained, in many cases, only through the simultaneous transfer of multiple embryos. In the United States, a mean number of greater than 2.5 embryos per patient are routinely transferred leading to overall multiple birth rates of more than 30%. In oocyte donation cycles, where the embryos should possess the highest reproductive potential, the transfer of more than one embryo leads to a multiple gestation rate of greater than 40%.

The risks related to multiple gestations are well known and include preterm delivery, low birth weight, and a dramatic increase in the relative risk for cerebral palsy (reviewed by [1]). These complications lead to a higher incidence of medical, perinatal and neonatal complications and a ten-fold increase in health care costs compared to a singleton delivery [2]. Decreasing the prevalence of multiple gestations while maintaining or improving overall pregnancy rates remains the most significant contemporary goal of infertility research.

In a number of countries, including Norway, Sweden, Denmark, Belgium, England, Italy, Germany and Australia, the dangers associated with multiple pregnancies have been allayed by legal restrictions on

the number of embryos that can be transferred in a single IVF cycle. For example, in most Scandinavian countries and Belgium the government has set a legal limit of single embryo transfer (i.e., only one embryo to be transferred per cycle) for specific patient groups, while many other European countries have restricted the number of transferred embryos to a maximum of two. In other parts of the world, where no legal restrictions exist, the onus is on the individual clinic (as well as the patient) to decrease the number of embryos transferred so that an acceptable balance can be achieved between the risks associated with multiple gestations and "acceptable" pregnancy rates. The current indications are that in the future clinics in the United States, and other countries currently lacking legislation, will be compelled via legal, financial and/or moral obligation to restrict the number of embryos transferred in order to minimize the risk of multiple gestations.

A major issue in limiting the number of embryos transferred is the apparent inability to accurately estimate the reproductive potential of individual embryos within a cohort of embryos using the existing selection techniques, which largely encompasses morphological evaluation. Faced with the scenario that we, the worldwide IVF community, will in the future have to select only one or two embryos for transfer, we will be forced to make certain choices. The first may be to rely on less aggressive stimulation protocols hence generating a lower number of eggs at collection [3]. Paradoxically, the generation of a smaller number of oocytes could lead to a greater percentage of viable embryos within a given cohort [4, 5]. The second choice is to improve the selection process for defining the quality of individual embryos so that the ones we choose for transfer are more likely to implant. This review will discuss one of the new emerging approaches in the search for biomarkers of embryo quality and its preliminary application in IVF.

Non-invasive Assessment of Embryo Culture Media

The inherent ease for the laboratory to assess various morphological markers makes it the preferred assessment technique to transfer embryos. Even with the adoption of more complex forms of assessment [6–9] it will still remain as one of the tools we have in our armoury for assessment. However, a number of quantitative techniques are now being optimized which can monitor the uptake of specific nutrients by the embryo from the surrounding medium, and to detect the secretion of specific metabolites and factors into the medium [10, 11]. To measure such changes in culture media the non-invasive assessment tools must fulfil a number of criteria so that they can be applicable in IVF clinics.

The problem for clinical IVF has always been:

1. The ability to measure the change without damaging the embryo
2. The ability to measure the change quickly.
3. The ability to measure the change consistently and accurately

Historically it is accepted that there is a relationship between metabolic parameters and embryo viability. In 1980, Renard *et al.* [12], observed that day-10 cattle blastocysts which had an elevated glucose uptake developed better, both in culture and *in vivo* after transfer than those blastocysts with a glucose uptake below this value. Subsequently, in 1987, using a relatively new technique of non-invasive microfluorescence, Gardner and Leese [13] measured glucose uptake by individual day-4 mouse blastocysts prior to transfer to recipient females. Those embryos that went to term had a significantly higher glucose uptake in culture than those embryos that failed to develop after transfer. This work was then built on by Lane and Gardner [14], who showed that glycolytic rate of mouse blastocysts could be used to select embryos for transfer prospectively. Morphologically identical mouse blastocysts with equivalent diameters were identified using metabolic criteria, as "viable" prior to transfer and had a fetal development of 80%. In contrast, the embryos that exhibited an abnormal metabolic profile (compared to *in vivo* developed controls), developed at a rate of only 6%. Clearly such data provides dramatic evidence that metabolic function is linked to embryo viability.

Only a few studies have been performed on nutrient uptake and the subsequent viability of the human embryo. In a retrospective analysis Conaghan *et al.* [15] observed an inverse relationship between pyruvate uptake by 2- to 8-cell embryos and subsequent pregnancy. In a study on human morulae and blastocysts of different degrees of expansion, no conclusive date was generated on the ability of nutrient consumption of utilization to predict pregnancy outcome Jones *et al.* [16]. Unfortunately, in both the above studies the medium used to assess embryo metabolism was a simple one, lacking lactate, amino acids and vitamins. Under such culture conditions the resultant stress on the embryos could have been detrimental, and

therefore it is questionable whether any meaningful data could have been obtained. In contrast, Van den Bergh *et al*. [17] showed that in patients who conceived following blastocyst transfer their embryos had an elevated glucose uptake and a higher oxidative rate compared to those blastocysts which failed to establish a pregnancy. Significantly, in the work of Van den Bergh *et al.* [17] a complete medium was used for the metabolic assessment, thereby alleviating the culture induced metabolic stress.

Furthermore, two studies have determined the relationship between embryo nutrition and subsequent development *in vitro* [18, 19]. Gardner *et al.* [18] determined that glucose consumption on day 4 by human embryos was twice as high in those embryos that went on to form blastocysts. Furthermore, it was determined that blastocyst quality affected glucose uptake. Poor quality blastocysts consumed significantly less glucose than top scoring embryos. In studies on amino acid turnover by human embryos, Houghton *et al.* [19] determined that alanine release into the surrounding medium on day 2 and day 3 was highest in those embryos that did not form blastocysts. Therefore, assessing metabolic activity and metabolic normality may prove to be a feasible way to determine human embryonic "health". To this end, Brison *et al.* [20] have reported that changes in concentration of amino acids in the spent medium of human zygotes cultured for 24 h in an embryo culture medium containing a mixture of amino acids using High Performance Liquid Chromotography. They found that aspargine, glycine and leucine were all significantly associated with clinical pregnancy and live birth.

Other techniques have also been reported to measure metabolic parameters in culture media however they have yet to be tested in a clinical IVF setting. These include the self-referencing electrophysiological technique, which is a non-invasive measurement of the physiology of individual cells and monitors the movement of ions and molecules between the cell and the surrounding media [21, 22]. One technique that is of the above mode is that which non-invasively measures oxygen consumption of developing embryos. Interestingly, although this technology has been shown to correlate with bovine blastocyst development it was less successful in predicting mouse embryo development [23, 24]. The technology has yet to be assessed in a clinical IVF setting however.

A number of studies have also investigated the assessment of secreted factors in the embryo culture media and correlated them with better embryo development and pregnancy rates. One such factor is soluble HLA-G [25, 26] which is believed to protect the developing embryo from destruction by the maternal immune response. Soluble HLA-G has been found in media surrounding the early embryo and a number of papers have also reported that its presence correlates with the improved pregnancy potential of an embryo [27–29]. Recently, some studies have raised some serious concerns regarding the use of HLA-G production as a marker of further developmental potential [30–32], and prospective clinical trials are needed to further evaluate this parameter. Included, in the studies examining secretion of factors in the media by embryos are numerous papers examining the secretion of platelet activating factor (PAF). The clinical utility of PAF in an IVF setting has also yet to be stringently examined (see review by O'neill [33]). Another indirect assay of soluble markers that may be present in embryo culture media was that described by Sakkas *et al.* [34] where it was determined that cell-free media from human embryos cultured to the blastocyst stage contained a soluble molecule that induced HOXA10 expression in an endometrial epithelial cell line (Ishikawa). Finally, a more direct analysis of protein markers in embryo culture media has been shown by Katz-Jaffe *et al*. [35], using proteomic based technology. They found differential protein expression profiles between early and expanded blastocysts, as well as between developing blastocysts and degenerate embryos.

Metabalomics

A new and emerging technology which may allow us in the future to measure factors in embryo culture media is metabolomics. The complete array of small-molecule metabolites that are found within a biological system constitutes the metabolome and reflects the functional phenotype [36]. Metabolomics is the systematic study of this dynamic inventory of metabolites, as small molecular biomarkers representing the functional phenotype in a biological system. Using various forms of spectral and analytical approaches, metabolomics attempts to determine metabolites associated with physiologic and pathologic states [37]. In relation to IVF, a wavelength specific genetic algorithm (Molecular Biometrics, USA) is used to determine a relative viability (Via Test-*E*™) score based on differences between transferred embryos which did or did not result in implantation (fetal heart detection). This is performed by measuring concentrations of key functional groups

Table 1. Metabolomic analysis resulting in viability scores (mean ± SD) calculated using Raman and NIR spectra of culture media for embryos that implanted and lead to delivery, and those that did not implant. For Raman mean values were calculated for culture media of embryos that implanted and lead to delivery (n=15), and those that did not implant (n=21). For NIR mean values were calculated for culture media of embryos that implanted and lead to delivery (n=16), and those that did not implant (n=17) (adapted from [38]).

	0% Implantation	100% Implantation	Significance
Raman Spectroscopy	0.326 ± 0.288	0.589 ± 0.222	P<0.01
NIR Spectroscopy	0.292 ± 0.224	0.671 ± 0.276	P<0.05

(for eg. -SH, C=C, -CH, -OH, and -NH groups), adjusted for parallel controls. Metabolic studies of embryos are beginning to indicate that embryos that result in pregnancy are different in their metabolomic profile compared to embryos that do not lead to pregnancies [38]. Investigation of the metabolome of embryos, as detected in the culture media they grow in, using targeted spectroscopic analysis and bioinformatics may therefore divulge these differences. In one study examining this technology, Seli *et al.* [38] established that differences between viable and non-viable embryos are detectable in the culture media using both Raman and Near Infrared (NIR) spectroscopy. Briefly, a total of 69 day 3 spent embryo culture media samples from 30 patients with known outcome (0 or 100% sustained implantation rates) were evaluated using Raman and/or NIR spectroscopy. A statistical formula was used to assign a relative "embryo viability score"—equating to embryo reproductive potential—and it was found that this score correlated to positive or negative implantation outcomes. Both Raman and NIR spectroscopic analysis of the spent culture media of embryos with proven reproductive potential demonstrated significantly higher viability scores than those that failed to implant (Table 1). Interestingly, when human embryos of similar morphology are examined using the same NIR spectral profile, their viability scores vary remarkably in relation to morphology indicating that the metabolome of embryos that look similar differ significantly. For example, the majority of embryos scored morphologically as excellent or good, display viability scores ranging anywhere between 0.15 and 0.6. This observation is in agreement with the study of Katz-Jaffe *et al*. [35, 39], who revealed that the proteome of individual human blastocysts of the same grade differed between embryos, again indicating that embryo morphology is not completely linked to its physiology.

In a concurrent metabolomic study to that above the individual idiosyncrasies of the spectral profiles of embryos that did and did not lead to pregnancy were used to create a statistical formula (genetic algorithm). The genetic algorithm established was subsequently used to predict the likelihood of pregnancy from blinded embryo culture media samples. When the model developed using NIR was used to test a subgroup of 16 day 3 embryo samples collected at a different center and cultured using a different type of commercial media, by observer blinded to pregnancy outcome, viability scores of embryos with proven reproductive potential were significantly higher compared to embryos that failed to implant [40]. A larger analysis of single embryo transfer cycles has also been undertaken in collaboration with the Kato Ladies Clinic whereby an NIR spectral analysis of frozen day 2 embryo culture media samples was performed blinded to outcome. Individual metabolic profiles were established from 7 μl of the samples with each measurement taking less than 1 minute. Statistical analysis performed on the metabolic profiles established a viability score (as generated above in the Seli *et al.* study [38]) that was significantly different (P<0.001) between the pregnant and non-pregnant patients. A cut off value for predicting pregnancy was taken at >0.3. When this cut off was used to examine embryos of excellent and good morphology that underwent single embryo transfer a significant difference was found in the establishment of pregnancy (Fig. 1). Although this technology needs to be further proven it does appear to fit the 3 key criteria necessary to be used routinely in a clinical IVF setting.

Conclusion

Analysis of embryo morphology and the development of suitable grading systems have assisted in the selection of human embryos for transfer. However, it is envisaged that in the near future morphology will also be significantly aided by a non-invasive analysis of

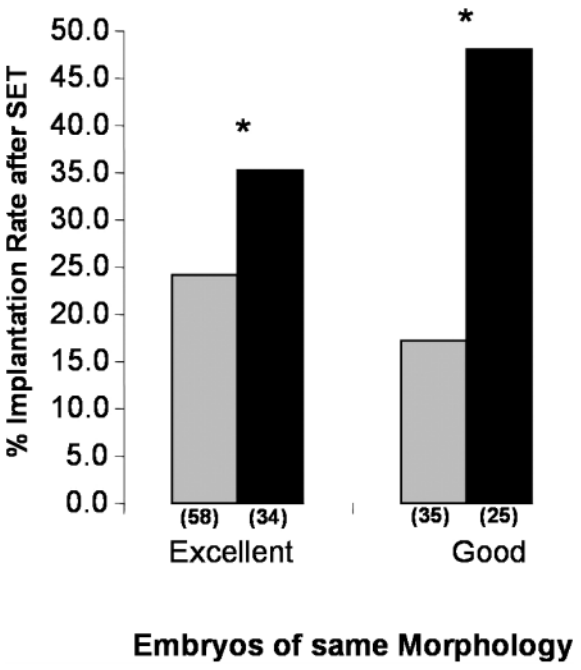

Fig. 1. Single embryo transfer implantation rates of day 2 embryos comparing embryos transferred with either an excellent or good morphology and a NIR Spectroscopy Via Test-*E*™ score of greater (black column) or less than 0.3 (grey column). The number of single embryo transfers performed are in parentheses (Data are from the Kato Ladies Clinic and Molecular Biometrics LLC) (*signifies significant $P<0.05$ difference).

biomarkers in the culture media that give a better reflection on embryo physiology and function. One such technique that holds great promise to be able to perform this assessment in a clinical IVF setting is metabolomics. The addition of this technology will be of immense value in helping both clinicians and embryologists to more confidently select the most viable embryos within a cohort and aid tremendously the move to single embryo transfers.

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