A crucial step in assisted reproduction technology: human embryo selection using metabolomic evaluation

We present a new methodology to predict embryo viability in assisted reproductive technology (ART) treatments by determining the relative amino acid concentrations in human embryo culture medium on day 3, using high-performance liquid chromatography with mass spectroscopy analysis without derivatization. The model was performed with soft independent modeling of class analogy for the samples from nonpregnancy and pregnancy cases. (Fertil Steril® 2010;94:772–4. ©2010 by American Society for Reproductive Medicine.)

Approximately three million children around the world have been born by means of IVF in the span of three decades [1]. There have been many studies to help couples undergoing assisted reproduction technology (ART) treatments achieve a higher success rate and a lower number of multiple pregnancies [1]. Nevertheless, research on human embryo development has, as yet, failed to provide a reliable, cost-effective, and efficient predictive test of embryo viability [2], and it has only been possible to apply morphological features.

More than 125,000 ART cycles per year are started in the United States, where ART accounts for approximately 1% of all births [3]. Unfortunately, the implantation rate remains low because only morphological criteria can be applied to evaluate the embryo's ability to implant. The highest success rates achieved with ART are attained with the transfer of two or more embryos, bringing the risk of multiple pregnancies [4], and consequently, a huge medical and social burden [5–8].

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At present IVF clinics are transferring fewer embryos [4, 8, 9], therefore there is an increasing reliance on the skill of the IVF laboratory's techniques to maximize the embryo implantation rate. Embryologists in IVF clinics use their skills to evaluate the quality of sperm, eggs, and embryos, and advise the doctors, nurses, and patients accordingly. The criteria for selecting which embryos will be transferred are based largely on cell number and their morphological appearance, which are relatively poor predictors of a successful implantation. More recently, the timing of embryo cleavage has also been included in the selection criteria [10]. Consequently, grading systems based on embryo cleavage and morphological features were developed [11–15], ling to significant improvements in implantation and pregnancy rates (PR), and fewer multiple gestations [16]. Unfortunately, the effectiveness of these techniques is still insufficient.

The limitations of evaluating embryos morphologically have led many researchers to look for additional technologies to assess the reproductive potential of a given embryo. Several metabolic parameters of developing embryos have been measured, using noninvasive techniques [3, 17–19], and in recent years various studies have shown the relationship between metabolites in the culture media and clinical pregnancy and live birth [20–26].

In this context we sought to develop a new skill that would help embryologists in their work, starting with one question and one hypothesis. Why embryons with good morphologycal characteristics lead to a failed implantation? Our hypothesis was founded on possible changes in the embryo culture medium during the 3 days before implantation. After 3 days the embryos were transferred into the patient, and it is reasonable to believe that changes in the culture medium may be related to the implantation potential for human embryos. The embryo needs amino acids to develop and synthesize new proteins. The consumption of amino acids present in the culture medium could be related to implantation capability. In our research we used a metabolomic approach to determine the relative concentration of amino acids in the culture medium by means of high-performance liquid chromatography with mass spectroscopy (HPLC-MS) and a classification chemometric tool (soft independent modeling of class analogy, SIMCA).

We studied 25 samples of the culture medium from human embryos after their implantation in 16 patients with known

outcome (0 or 100% ongoing PRs). Fifteen of the embryos failed to implant, and 10 were implanted successfully. Two of the 10 implantations with 100% ongoing PRs were single embryo transfers, and the other 8 were double embryo transfers. All of the implantations with 0 ongoing PRs were double embryo transfers, but only in one case we used the medium from one embryo in the HPLC-MS analysis (the medium from the other embryo was contaminated after the transfer). One fertilization cycle was completed before pregnancy confirmation. After 8 weeks, the pregnancy was confirmed by ultrasound scan (positive fetal heart). The infertility treatments were done at the Instituto Bernabeu in Alicante, Spain, in the first semester of 2009, using standard long luteal clinical protocols after taking oral contraceptives (OC) for 1 month. The protocol includes pituitary desensitization with an agonist and ovarian stimulation with a combination of recombinant human FSH and hMG. Ovarian response was monitored by transvaginal ultrasound (TVUS) and E_2 concentration. Ovulation was induced with 250 μg of recombinant hCG. Under sedation, oocytes were aspirated by a TVUS-guided needle 36 hours after hCG administration. On day 1, embryos fertilized with two pronuclei were placed into individual 50-μL drops of G1.3 culture medium (Vitrolife, Goteborg, Sweden), under standard incubation conditions to the cleavage stage and then transferred into the patients on day 3. After the embryos were transferred, the culture medium was obtained from the clinic and used in the analysis. Twenty microliters of culture medium, 20 μL of Met 0.1 mM as internal standard, and 60 μL of solvent A (30 mM ammonium acetate and 5% acetic acid at a ratio of 12.5:87.5, pH 2.5) were mixed in a tube and microinjected in the HPLC-MS system. The liquid chromatography coupled to tandem mass spectrometry with electrospray ionization (LC-ESI-MS-MS) analysis was performed as described (27), with modifications as described later. The LC-ESI-MS-MS analysis was performed with the Agilent (Santa Clara, CA) 1100 series HPLC instrument. The LC system was coupled with the Agilent 1100 Series LC/MSD Trap SL, with the possibility of carrying out MS/MS analysis. The mass spectrometer was operated in the positive ESI mode, and the ion spray voltage was set at 4 kV. Nitrogen was used as the sheath gas (30 psi), and the ion transfer capillary was heated to 350°C. Injections were carried out using an HTC Pal autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 20-μL sample loop. The MS-MS parameters of the amino acids were determined by analyzing the flow injection of amino acid standard solutions using the built-in syringe pump. The culture medium solutions were infused into the flow of the HPLC system through a T connection, under the following conditions: flow rate of the syringe pump, 10 μL/min; flow rate of the HPLC system, 0.3 mL/min (30 mM ammonium acetate and 5% acetic acid at a ratio of 12.5:87.5, pH 2.5, solvent A). The LC separations were carried out with Phenomenex (Torrance, CA) Luna 5μ SCX 100 Å column, 150-mm × 2.0-mm internal diameter, at 25°C. For the elution of the amino acids, an isocratic step was programmed with solvent A for 15 minutes. The overall flow rate was adjusted to 0.3 mL/min. Before use, the new SCX column was flushed overnight with 150 mM of ammonium acetate solution. We determined the retention time for individual amino acids using standard solution at the concentration of G1 culture medium (Vitrolife) by HPLC-MS/MS, to avoid ambiguity (27). The areas of the HPLC-MS peaks with the same retention times as the calibration standard were integrated and subsequently used for calculating

the concentrations of each amino acid. To calculate the relative amino acid concentration, the peak area was divided by the peak area of the internal standard. An exploratory analysis of the data (box plots were made with SPSS v 15.0; SPSS Inc., Chicago, IL; data not shown) suggests that amino acid consumption was higher in the culture medium of the embryos that resulted in a pregnancy. Brison and colleagues (21) also reported that noninvasive analysis of amino acid turnover has the potential to improve significantly the selection of the most viable embryos in an IVF cycle. The differences between the relative amino acid concentration in the culture medium from embryos resulting in pregnancy and those that do not are clear. It is difficult or impossible to view the data grouping in seven dimensions (one dimension for each amino acid). A multidimensional region method is needed to determine where a sample point is expected to be located. Principal component analysis (PCA) is the perfect tool for this task (28).

The SIMCA is a classification algorithm based on producing a PCA model for each class of samples and then comparing their distance from the class confidence limits (28). If a point lies within that confidence limit, it is called a member of the class. This method has the additional advantage that a sample can be a member of one or more classes, as well as of no class. The closest class can also be calculated for samples that are not a member of any class. The reason for developing a classification scheme based on relative amino acid concentration was to create a classification method that was not based on abstract regression vectors, but rather on chemically interpretable data, and where data deemed to be interfering could be removed from consideration. The SIMCA was performed on the samples from nonpregnancy and pregnancy cases. Each case was modeled with a PCA model of 2-3 principal components (as required to capture ≥99% of the

FIGURE 1

Coomans plot and model distance plot in SIMCA model. Nonpregnancy (*open circles*) and pregnancy (*closed circles*) samples. Pregnancy was referred to the success of the implantation of a single embryo determined by the positive fetal heart after 8 weeks. In several cases two embryos were implanted (see text). Cases in which two embryos were implanted and only one positive fetal heart was found were not included in the present study.

SIMCA model, Significance = 25.0% Model 1: Non-pregnancy, Model 2: Pregnancy
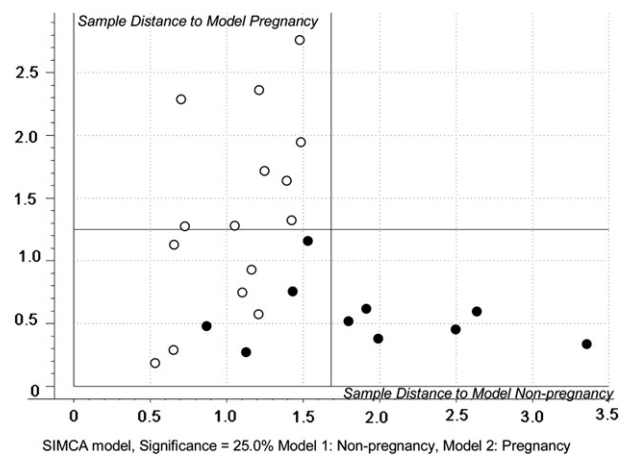
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variance) and the model distance was found for each sample to the model of each of the three locations. A personal computer with The Unscrambler 9.8 (Camo, Oslo, Norway) was used for this purpose.

Because each class has its own distribution, the distance of each sample from each class must be calculated. This should not be surprising, as these samples were used as the training set. The most significant result is that no sample from any other case in the set is classified incorrectly in another class. Therefore, the embryos with a high implantation rate (pregnancy) are in one group and, as a more important practical application, the embryos with a low implantation rate (nonpregnancy) are in another group (Fig. 1). The PCA models are based on the samples from a known class. This means that samples that had the same relative amino acid concentrations would still be classified within those with the same case. In addition, using SIMCA, a sample can be a member of two or more classes, or a member of none of the classes modeled (Fig. 1). With all of these considerations in mind, the near-zero error classification is impressive, and lends credibility to the postulate that there are real relative amino acid concentration differences between samples that can be attributed to nonpregnancy and pregnancy cases.

After three decades of human embryo evaluation based only on morphological criteria these results have shown that a classification method based on chemically interpretable amino acid concentrations determined by HPLC-MS analysis is possible. It shows that there are differences in the relative amino acid concentrations in the culture medium from embryos with differing implantation potential. The success of a method that couples HPLC-MS with SIMCA is not specific to this application, but applicable to any spectroscopy or multiway data-based classification such as infrared fingerprinting. More studies using this method are needed to measure the relative amino acid concentrations that occur for different embryo culture medium preparations, and to compare the differences between samples from embryos that fail to implant and those that result in pregnancy.

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