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Original Article

Non-Invasive Prediction of Blastocyst Formation by Day Three Embryo Culture Medium Mass Spectrometry Lipid Fingerprinting

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ABSTRACT

Objective: To identify lipid markers of blastocyst formation by day three culture medium mass spectrometry (MS) fingerprinting.

Methods: For this study, 50 embryo samples from culture media were harvested on day three, from patients undergoing embryo transfers on day five. Samples were split into groups based on their degree of expansion and hatching status on day five (Complete-Blastocyst, n=25 and No-Blastocyst, n=25) and its secretomes were analysed by MS. Mass spectra fingerprinting was acquired using a Q-Tof spectrometer (LC-MS, Agilent 6550 iFunnel Q-TOF) equipped with an automated injector. The data was analysed using the principal component analysis (PCA) followed by a partial least square discrimination analysis (PLS-DA), combined with variable influence in the projection (VIP) scores.

Results: In total, there were 1,657 ions found, in which 165 ions were differently expressed between groups, with a fold chance ≥ 4x and $P<0.001$, in the t-test. PLS-DA showed a clear separation between the groups and among 15 VIPs selected by the program, 13 of them were highly expressed in the Complete-Blastocyst Group and two were expressed in the No-Blastocyts Group. Besides embryo status on day five, the PLS-DA was also able to classify samples according to patients' age. Lipids supposedly highly expressed in the Complete-Blastocyst Group included: isoprenoids, diacylglycerols, sterols, fatty esters, secosteroids, phosphosphingolipids, glycerophosphates and diacylglycerophosphates, while fatty amides were suggested to be highly expressed in the No-Blastocysts Group.

Conclusions: Day three culture medium MS is a promising approach for the identification of embryos that should be cultured until day five.

Keywords: Lipid, Fingerprinting, Embryo, Blastocyst, Mass Spectrometry

INTRODUCTION

Although more than two million children have already been born as a consequence of assisted reproductive technologies (ART), around 70-80% of in vitro produced embryos fail to implant, and 66% in vitro fertilization (IVF) cycles fail to result in pregnancy (Assou *et al*., 2011; Seli *et al.,* 2004; Patrizio and Sakkas, 2009). The identification of factors that may influence the implantation of in vitro-produced embryos is one of the most investigated fields in ART. Although high-quality embryos may be available for transfer, choosing the best embryo for transfer has become a major challenge in IVF.

Serial observation of embryo morphology, which is a common technique for embryo evaluation, has been considered to be a key predictor of implantation and pregnancy (Balaban *et al*., 2001; Lundin *et al*., 2001; Lan *et al*. 2003; Nagy *et al*., 2003; Payne *et al*., 2005; Holte *et al.,* 2007; Racowsky *et al*., 2010; Ahlstrom *et al.,* 2011; Alpha Scientists, 2011; Aydin *et al*., 2011). However, it has been suggested that embryo morphology is insufficient for predicting successful implantation (Katz-Jaffe *et al*., 2009; Assou *et al*., 2011; Mastenbroek *et al*., 2011). In addition, this method is highly subjective (Paternot *et al*., 2009;Filho *et al*., 2010).

Prolonging the embryo culture period allows for a better selection of embryos for transfer because laboratory assessment is performed after the embryonic genome has begun to be expressed (Tesarik *et al*., 1988). However, because of our continuing inability to predict which cleavage-stage embryos will develop into viable blastocysts (Schoolcraft and Gardner, 2001; Westphal *et al*., 2003), assisted reproduction centres are reluctant to adopt extended embryo culture to avoid embryo transfer cancellations (Sepulveda *et al*., 2011).

In the post-genomic era, many "omics" efforts are being focused on understanding the relationships between genome, DNA transcripts, proteins, metabolites and phenotypes in cells and organisms (Katz-Jaffe *et al.,* 2009). Non-invasive approaches for embryonic development potential have the advantage of increasing the knowledge of embryo physiology, therefore enabling the development of methods to predict developmental competence and viability (Hamel *et al*., 2008). These approaches include genomic and proteomic profiling, embryonic metabolome analytical examination (Botros *et al.,* 2008; Bromer and Seli, 2008; Katz-Jaffe *et al*., 2009; Aydiner *et al*., 2010; Ferreira *et al.,* 2010; Seli *et al*., 2010; Cortezzi *et al*., 2011), and most recently: lipidomics (Quehenberger *et al.,* 2010).

Mass spectrometry (MS) fingerprinting has been shown to provide a reliable approach to evaluate culture media profiles (Ferreira *et al*., 2009). MS fingerprinting has been applied to evaluate embryo proteome and metabolome, with promising results (Katz-Jaffe *et al.,* 2006; Cortezzi *et al.,* 2011; D'Alessandro *et al*., 2012; Lagarrigue *et al.,* 2012; Sanchez-Ribas *et al*., 2012; Cortezzi *et al*., 2013).

Therefore, the goal for the present study is to utilise the analytical power of MS with minimal sample preparation and minute analysis to identify possible lipid markers of embryo developmental competence in the surrounding culture medium.

MATERIAL AND METHODS

Experimental Design

For this study, 50 culture media samples were harvest on day three, from patients undergoing day five embryo transfers. Embryos were split into groups based on their degree of expansion and hatching status on day five (Complete-Blastocyst, n=25 and No-Blastocyst, *n*=25) and their secretomes were analysed by MS.

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The patients signed an informed consent form, in which they agreed to share the outcomes of their cycles for research purposes. The local institutional review board approved the study.

Controlled Ovarian Stimulation and Oocyte Retrieval

Controlled ovarian stimulation was achieved by using recombinant FSH (Gonal-F; Serono, Geneva, Switzerland), as a daily dose, starting on day three of the cycle. Pituitary blockage was performed by using a GnRH antagonist (Cetrotide, Serono, Geneva, Switzerland), starting when at least one follicle ≥14 mm was visualised.

Follicular growth was monitored using transvaginal ultrasound examination starting on day four of the gonadotropin administration. When adequate follicular growth and serum 17β estradiol levels were observed, recombinant hCG (Ovidrel; Serono, Geneva, Switzerland) was administered to trigger the final follicular maturation. The oocytes were collected 35 hours after hCG administration through transvaginal ultrasound ovum pick-up.

Preparation of Oocytes and Morphology Assessment

Retrieved oocytes were maintained in culture medium (Global® for Fertilization, LifeGlobal, Connecticut, USA) supplemented with 10% Human Synthetic Albumin (HSA, Irvine Scientific, Santa Ana, USA), covered with mineral oil (Ovoil™ - Vitrolife, Kungsbacka, Sweden) at 37°C and 6% CO2 for 5 hours. Surrounding cumulus cells were removed with exposure to a HEPES buffered-medium containing hyaluronidase (80 IU/mL, Irvine Scientific, Santa Ana, USA). The remaining cumulus cells were then mechanically removed by gentle pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, Virginia, USA). The oocytes were checked for oocyte maturation and those which had released the first polar body (metaphase II oocytes – MII) were considered mature and used for ICSI.

Intracytoplasmic Sperm Injection

Intracytoplasmic Sperm Injection was performed on all MII oocytes using the technique described by Palermo *et al.* (1992). The oocytes were individually placed in 4-µL droplets of buffered medium (Global® w/HEPES, LifeGlobal, Connecticut, USA), and sperm was placed in a central 4-µL droplet of polyvinylpyrrolidone solution (PVP, Irvine Scientific, Santa Ana, USA) in a 50 X 40-mm glass culture dish (WillCo-dish®, New Jersey, USA) covered with warm mineral oil (Ovoil™, Vitrolife, Kungsbacka, Sweden), on a heated stage (37.0 ± 0.5°C) of an inverted microscope.

Fertilization, Embryo Quality and Embryo Transfer Assessments

After ICSI, the presumptive embryos were individually maintained in a 50-µL drop of culture medium (Global®, LifeGlobal, Connecticut, USA) supplemented with 10% human serum albumin (HSA) and covered with mineral oil in a humidified atmosphere with 6% CO2 at 37 °C until transferred - which occurred on the fifty day of development.

Approximately 18h after ICSI, fertilization was confirmed by the presence of two pronuclei and the extrusion of the second polar body. Subsequently, embryos were transferred to new drops of culture medium to be individually cultured for 48 hours. On day three, the culture media was refreshed and the embryos were transferred to another dish and cultured until day five.

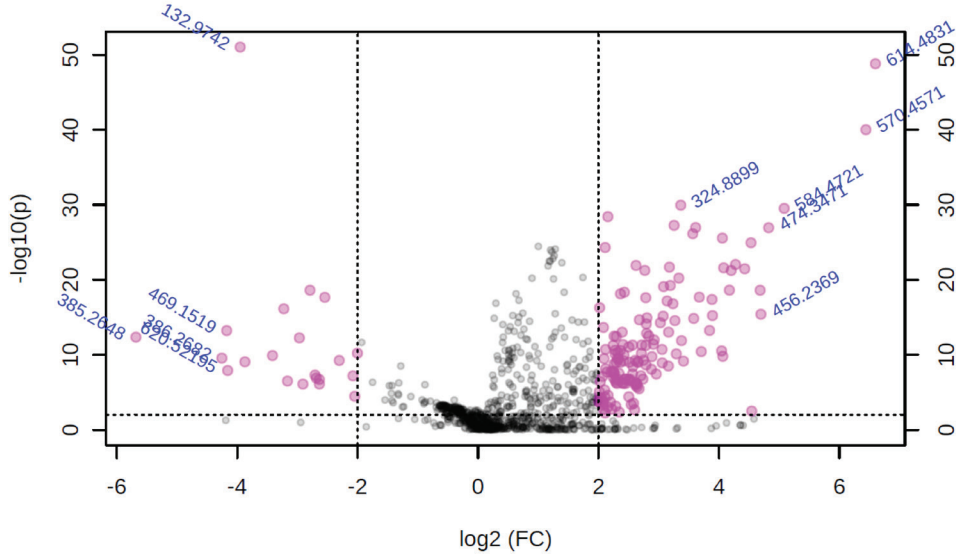
For the blastocyst stage morphology, the following characteristics were recorded: developmental degree and hatching, status, size and compactness of the ICM, cohesiveness and number of TE cells. Briefly, embryos were given a numerical score from one to six on the basis of their degree of expansion and hatching status, as follows: 1, an early blastocyst with blastocoels that occupied less than half the volume of the embryos; 2, a blastocyst with a blastocoel that was greater than half the volume of the embryo; 3, a full blastocyst with blastocoels completely filling the embryo; 4, an expanded blastocyst; 5, hatching blastocyst; and 6, a hatched blastocyst. Full blastocysts onward were considered for the Complete-Blastocyst Group, the other embryos were considered for the Non-Blastocyst Group.

Sample Preparation, Mass Spectrometry Analysis and Data Analysis

On day three of embryo development, the culture media was individually collected and stored at -20 °C, while embryos were moved to another culture dish with refreshed culture medium until the day of transfer.

The lipids from culture medium were individually extracted using the Bligh and Dyer method (Bligh & Dyer, 1959), dried and diluted in 400 µL of MeOH. Mass spectra

Figure 1: Volcano Plot Graphic showing ions differentially expressed between Complete-Blastocyts and No-Blastocysts groups, with a fold chance ≥ 4x and *P*<0.001, in the t test.



were obtained with a direct infusion of both the negative and positive ion modes into a Q-Tof mass spectrometer (LC-MS, Agilent 6550 iFunnel Q-TOF) equipped with an automated injector. The data was analysed using the principal component analysis (PCA), followed by a partial least square discrimination analysis (PLS-DA), combined with variable influence in the projection (VIP) scores. The statistical analysis was performed using the Metabo-Analyst 2.0 (http://www.metaboanalyst.ca).

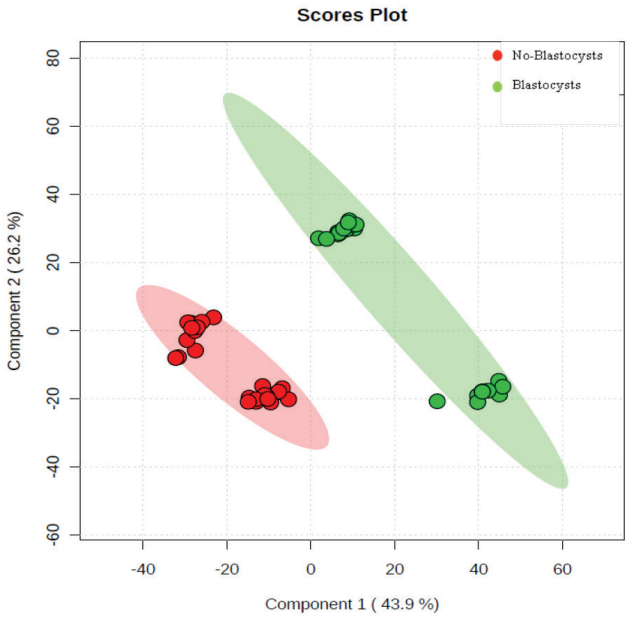
RESULTS

Overall, 1,657 ions were found. When the univariate analysis was performed, 165 ions were found to be differentially expressed between the groups, with a fold likelihood ≥ 4x and $P<0.001$, in the t-test (Figure 1).

PLS-DA showed a clear separation between the Complete-Blastocyst and Non-Blastocyst groups (Figure 2). As shown in figure 2, besides the embryo status on day five, the samples were split by the program into two more groups. When these samples were analysed, we noted that the MS lipid fingerprinting also correlated with the patient's age. The PLS-DA showed a separation between samples obtained from patients >38 years old and those ≤ 37 years old.

Among 15 VIPs selected by the program, 13 of them

Figure 2: A Pls-Da plot of the scores for samples from the Complete-Blastocysts and No-Blastocyts groups.



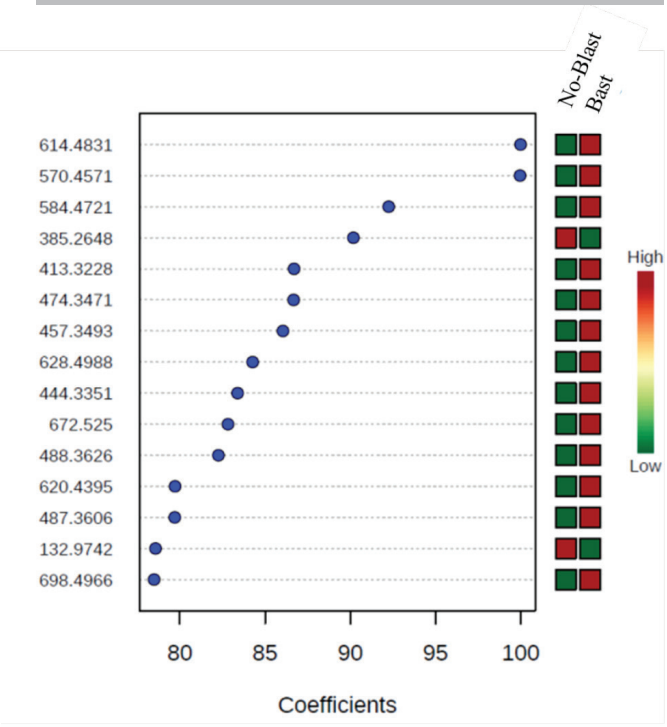
were highly expressed in the Complete-Blastocyst Group and 2 in the Non-Blastocyst Group (Figure 3).

The lipids supposedly highly-expressed in the Complete-Blastocyst Group included isoprenoids, diacylglycerols, sterols, fatty esters, secosteroids, phosphosphingolipids, glycerophosphates and diacylglycerophosphates, while fatty amides were suggested to be highly expressed in the Non-Blastocysts Group.

DISCUSSION

Modern approaches for lipidomics are dominated by MS, commonly preceded by separation by liquid chromatography or gas chromatography. The novel MS-based lipidomics afford the study of intact lipid molecular species

Figure 3: The most important VIP ions for separating the groups Complete-Blastocyst and No-Blastocyst groups.



from very small amounts of samples, and such methods, due to their wide dynamic range, enable quantitative or relative determination of compounds across a broad range of concentrations. Therefore, as an emerging "omics" field, lipidomics provides a powerful approach to understanding cellular biology (Quehenberger *et al*., 2010) and integrate the investigation of the role of genomics, transcriptomics, proteomics, and metabolomics in cell function.

To the best of our knowledge, in the present study, possible lipid biomarkers for blastocyst formation competence were suggested. By means of the PLS-DA loading analysis, which identifies the most important ions for separating the two groups based on their m/z values, we were able to observe several ions with higher potential for acting as biomarkers. Not only the presence or absence of specific ions but also their relative abundance in the spectra allowed separation between the complete and no-blastocyst groups.

Recently, the "freeze-all' policy has emerged as an alternative to fresh embryo transfer, to improve IVF outcomes (Shapiro *et al*., 2011a; Shapiro *et al*., 2011b; Roque *et al*., 2013). In this method, the entire cohort of embryos is cryopreserved, and embryo transfer is performed later in a natural cycle, or in a cycle with hormonal replacement for endometrial priming (Roque *et al.*, 2015). The potential advantage of this method is that it provides a more physiologic environment in which embryo transfer occurs; this approach could lead to better pregnancy rates and decrease maternal and perinatal morbidity (Barnhart, 2014). However, the patient's selection and the cost for cryopreservation are potential downsides of this technique. Alternatively, the best embryos can be cryopreserved and transferred into a more receptive endometrium while the other embryo may be transferred in the fresh cycle. Therefore, the identification of possible biomarkers of blastocyst formation would be crucial for the implementation of embryos cryopreservation as a routine procedure, avoiding the deleterious effects of controlled ovarian stimulation on endometrium receptivity.

Moreover, the identification of patients able to benefit from extended embryo culture programs would be imperative for assisted reproduction success. In fact, although it is known that prolonging the embryo culture period enables a better selection of embryos for transfer, leading to increased implantation rates and reduced risk of twins and higher order pregnancies (Forman *et al*., 2013), there is concern that a strategy of blastocyst culture may result in higher cycle cancellation rates (Glujovsky *et al*., 2012).

Many metabolic parameters of developing embryos have been studied using a variety of non-invasive methods (Sakkas & Gardner, 2005). These studies demonstrate an underlying molecule expression difference between embryos that converts into blastocysts and those that do not, and this lays the groundworks of a metabolomic approach in the assessment of embryo viability.

Gardner *et al*. (2001) reported that glucose uptake was larger in human blastocysts of higher grade, whereas Brison *et al.* (2004) found that elevated asparagine, and decreased glycine and leucine levels in embryo culture media correlate with pregnancy. These and other studies suggest that embryos with positive and negative reproductive potential alter their environment differently and this is reflected in the surrounding metabolites (Scott *et al*., 2008; Seli *et al*., 2008; Vergouw *et al*., 2008; Seli *et al*., 2010; Ahlstrom *et al*., 2011b; Sfontouris *et al*., 2013).

On these previous studies, metabolomic profiling of spent embryo culture media was mainly performed using spectroscopy. In the present study, a different method for embryonic evaluation, the MS, was applied. Mass Spectrometry allied with PLS-DA model has been successfully employed in biomarker identification in other areas than reproduction (Duan *et al*., 2011; Wang *et al*., 2012a; Wang *et al.,* 2012b; Yang *et al*., 2012). Recently, our group also applied an embryo viability prediction (Cortezzi *et al*., 2013).

In conclusion, our findings demonstrated that the day three culture medium MS may identify possible lipid biomarkers of embryos which are able to convert into blastocysts; therefore, this may be a promising approach for the identification of embryos that should be cultured until day five or even cryopreserved and transferred latter into a more receptive endometrium.

CONFLICT OF INTERESTS

No conflict of interest have been declared.

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