Zygote serine decreased uptake from the fertilization medium is associated with implantation and pregnancy

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Abstract

Purpose To identify an association between amino acids (AAs) metabolism and reproductive outcome.

Methods Prospective collection, observational study, in patients undergoing fresh, double embryo transfer (ET), in a tertiary hospital referral IVF unit. Spent day 1 and day 3 media were collected. Concentrations of taurine, aspartic acid, proline, and serine in the medium were determined using a liquid-chromatography mass-spectrometer (LCMS/MS). Data was analyzed according to excretion versus uptake, and a cut-off value was calculated based on a receiver operating curve (ROC). Pregnancy rates were also calculated after stratification into subgroups in accordance with AA metabolism.

Results Seven out of 19 patients conceived (36.8 %). The ORs for pregnancy when the zygotes secreted aspartic acid, serine and proline above the cut-off value were 2.9, 5.67 and 5.21 ($p < 0.05$). When both transferred embryos were above the cut-off value of serine the PR's were 62.5 %, 12.5 % when both were below, and 33.3 % when one was above and the other below ($p = 0.04$). Similar results were obtained for proline; PR's were 66.7, 18.7 and 28.6 % respectively, but with a borderline statistical significance ($p = 0.08$). The same trend was observed

in the case of aspartic acid but not near statistical significance. No differences in PRs were found in association with taurine turnover during fertilization or any of the studied AAs during the cleavage stage. There was no correlation between zygote or embryo AAs metabolism and embryo morphology.

Conclusions Serine and possibly proline decreased uptake from the fertilization medium is associated with pregnancy and might be useful for embryo selection.

Keywords In vitro fertilization · Zygote · Amino acid metabolism · Pregnancy

Introduction

Improving success rates while preventing multiple gestations has become the major objective in contemporary Assisted Reproductive Technology (ART). The achievement of this goal requires the development of objectively reliable, reproducible and robust technology to select the single best embryo for transfer. Currently, the conventional method of embryo grading is based on morphological parameters: the number of blastomeres and degree of fragmentation. It has been suggested that morphology alone has limited value in predicting an embryo's reproductive potential [1–4]. Furthermore, morphology rarely discriminates between euploid and aneuploid embryos [5, 6].

Given these limitations, new methods have emerged in an attempt to base embryo selection on quantifiable biological parameters more strongly associated with implantation, rather than plain morphology. These technologies are based on a variety of approaches and are under extensive research. Applying the principles of metabolomics, the measurement of small molecules associated with the physiological or pathological functions of a system, the

Capsule Zygote Serine metabolism is a potential biomarker for implantation and pregnancy.

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embryo and the culture medium are a system, and metabolites or molecules such as oxygen, glycolytic intermediates and amino acids are measured. Metabolomics can also be used for other biological environments in ART including follicles [7–9].

The embryo's microenvironment reveals the functional status of the embryonic system and is useful for embryo profiling. Studies that analyzed the metabolomes demonstrated a correlation between changes of culture media metabolites and the embryo reproductive potential. Measurements of pyruvate and glucose, the main energy sources of the embryo, were found to correlate with embryo viability and were predictive of live birth [10–12]. Oxygen consumption is a quality marker for human oocyte competence conditioned by ovarian stimulation regimens [13] that defines biochemical predictors of viability, quality and functionality. Amino acids turnover is predictive of pregnancy, blastocyst development, and chromosomal abnormalities [14, 15, 5]. Various analytical techniques such as spectroscopy/spectrometry and chromatography with different metabolomics models have been proposed for the measurement of these components.

The aim of this study was to develop a reliable method for embryo selection by identifying an association between changes in concentrations of amino acids in culture media and the embryos' implantation potential. These changes were measured by liquid chromatography tandem mass spectrometry (LCMS/MS).

Triple quadrupole mass spectrometry has the capacity for high sensitivity (e.g. $< 10^{-12}$ mol) metabolite detection. MS analysis involves the production of ions and the separation by their m/z ratio. In this study, we optimized an LCMS device for detection of minute changes in amino acid concentrations in the IVF culture media. The precursor ions were selected in the first quadrupole mass analyzer and fragmented in the collision cell followed by detection of the product ions in the second mass analyzer. The triple-quadrupole mass spectrometer was operated in the positive ionization mode. Detection and quantification was performed using multiple reaction monitoring (MRM). Ion-pairing reversed-phase liquid chromatography was implemented in this work. The addition of separation methods, such as liquid chromatography, simplifies sample preparation since it can function in any temperature and does not require sample volatility [16].

We focused on four amino-acids: serine, proline, aspartic acid, and taurine, as potential biomarkers for reproduction and investigated their turnover during both oocyte fertilization and cleavage stage culture. Unlike spectrometric methods that examine the spent medium globally utilizing patented devices that did not prove themselves in practice, the method we present is highly accurate, generic and is easily reproducible with any LCMS device.

Materials and methods

Patient population

IVF patients of varying ages, infertility etiologies, and differing embryo qualities undergoing fresh embryo transfer at the Hadassah Ein Kerem IVF unit were included. All female patients had a baseline FSH ≤ 15 IU/L. Only embryos resulting from ICSI were included to ensure that culture drops were not contaminated with other cells. Excluded from the study were cases with severe male factor ($< 1 \times 10^6$ total motile concentration), structural anomaly of the uterus, or requiring PGD. A standard embryo scoring system based on morphology and cleavage rate [17] was used for embryo evaluation and selection. Accordingly, a day 3 high quality embryo (grade A) was defined as an embryo that had ≥ 7 cells with less than 5 % fragmentation; grade B 5–20 % fragmentation, and grade C—20–50 % fragmentation. The study included the collection and analysis of discarded samples from spent media droplets. The media samples were cryopreserved and analyzed after all IVF cycles were completed. Since embryo selection for transfer was performed based on conventional clinical and embryologic parameters and there was no alteration in clinical care the, study was exempt from Ethical Committee approval.

Sample collection and preparation

The culture medium used after the performance of ICSI through day 1 was Quinn's Advantage™ Fertilization (HTF) Medium. On day 1 the medium was changed to Quinn's Advantage® Cleavage Medium (Sage media, Cooper Surgical, USA) and used through day 3.

Day 1 and cleavage stage samples of spent media were separately collected from each zygote and embryo. After removal of the zygote or embryo from the culture dish, a sample of 35 microliters of spent medium was placed into labeled vials and stored at -80°C . In parallel, unused medium droplets from the same bottle were incubated in identical conditions, stored and used as controls for comparison and assessment of alterations in the media.

After thawing for 30 min at room temperature, 20 microliters of medium from the bottom of the vial was transferred to an empty vial. To avoid contamination of the oil phase that coats the medium droplets in the incubator, 0.5 mL of distilled water and 0.5 mL of hexane were added to each vial and centrifuged for 2 min at 1200 RPM. The hexane dissolved any oil contaminating the medium and was separated from the watery phase by centrifugation. A sample of 180 microliters of the residue (culture media diluted by distilled water) was then transferred for LCMS/MS measurements.

Liquid chromatography—Mass spectrometry analysis

Instruments

The HPLC-MS/MS system (Thermo Scientific, San Jose, CA, USA) included an Accela Pump with a degasser module and an Accela Autosampler connected to a TSQ Quantum Access Max mass spectrometer via a heated electrospray ionization (H-ESI) interface.

HPLC conditions

Ion-pairing reversed-phase liquid chromatography was the chromatographic technique utilized with heptafluorobutyric acid (HFBA) as the ion-pairing reagent. Separations were performed on a Hypersil Gold C8 (Thermo Scientific), 5 μ m, 150 $\times$ 4.6 mm equipped with a Hypersil Gold C8 guard column (5 μ m, 10 $\times$ 4 mm). The chromatographic separation was achieved using a gradient program at a constant flow rate of 1 mL/min over a total run time of 8 min. An outline of the mobile phase gradient program is summarized in Supplementary Table 1. Chromatographic oven temperature was set at 30 °C and the autosampler tray temperature was maintained at 5 °C. Samples (5 μ L) were injected on to the column via a partial loop injection system and the needle was washed with 1 mL of methanol: water, 50:50, v/v, between injections. The first and last 2 min of the column effluent were diverted to waste.

MS/MS conditions

The triple quadrupole mass spectrometer was operated in positive ionization mode; detection and quantification were performed using multiple reaction monitoring (MRM). The high-purity nitrogen gas (15 L min⁻¹), used as sheath and auxiliary gases, was generated using a LCMS15-1 Parker nitrogen generator (Parker Hannifin Ltd., Gateshead, Tyne and Wear, England). 99.999 % pure argon (Moshalion, Jerusalem, Israel) was used as collision gas (1.0 mTorr). The pressure of the nitrogen sheath gas and auxiliary gas were set at 10 and 2 (arbitrary units). The ionization spray voltage, capillary transfer tube temperature and skimmer offset were set at 5 kV, 300 °C and 6 V, respectively. The vaporizing

temperature within the H-ESI source was maintained at 200 °C. The scan time was 0.05 s, with a scan width of 0.1 m/z and Q1 (first quadrupole) and Q3 (third quadrupole) peak width of 0.7. TSQ Tune Software (Thermo Scientific, San Jose, CA, USA) was used for the optimization of tuning parameters. Data acquisition and processing were carried out using the Xcalibur program (Thermo Scientific, San Jose, CA, USA). Compound specific parameters including the tube lens and the collision energy (CE) were optimized for a maximum of three transitions per amino acid and are shown in Supplementary Table 2.

HPLC and MS/MS conditions were standardized using solutions containing the studied amino acids in various concentrations dissolved in HPLC grade water.

Statistical analysis

The area of the chromatographic peak of each amino acid (Supplementary Fig 1), measured by LCMS/MS, represents the amino acid concentration. The measurements of taurine, aspartic acid, proline, and serine were subtracted from the results of the control medium droplet cultured and measured in parallel. The data were analyzed according to the uptake status of the studied AAs in comparison to the control sample. A ROC (receiver operator characteristic) curve was calculated for each amino acid and medium (fertilization and cleavage media) according to the LCMS results. A cut-off value was determined according to the nearest point to the optimum on a ROC curve. The pregnancy rate (PR) was calculated based on ongoing intrauterine pregnancies only. To address the issue of double embryo transfers, we stratified the results for each amino acid according to whether both embryos were above, below or on both sides of the calculated cut-off value, and separately calculated the PR for each subgroup. *P* values were calculated using the Chi-square linear by linear association test. This test is more powerful for ordered variables.

Results

Spent zygote and embryo culture media from 19 women undergoing IVF cycles were obtained and evaluated using LCMS/MS. Patient age was 35.75 $\pm$ 4.73 years, and

Table 1 Double ET pregnancy rates and zygote amino acid metabolism

Amino acid metabolic pattern	Serine pregnancies/ETs (%)	Proline pregnancies/ETs (%)	Aspartic acid pregnancies/ETs (%)
Both zygotes above threshold	5/8 (62.5)	4/6 (66.7)	2/3 (66.7)
One zygote above, one below threshold	1/3 (33.3)	2/7 (28.6)	3/7 (42.9)
Both zygotes below threshold	1/8 (12.5)	1/6 (16.7)	2/9 (22.2)
<i>P</i> value	0.04	0.08	NS

unexplained infertility was the most common infertility diagnosis. Each patient had two fresh embryos transferred, selection was based on morphological criteria, and surplus embryos were cryopreserved. Pregnancy rate was 36.8 %. The data of the fresh embryos transferred were analyzed according to excretion versus uptake of the four amino acids in regard to the cut-off value defined by the ROC curve.

Decreased uptake of serine and proline into the fertilization medium (day 1) were found to be highly associated with pregnancy. For zygotes with decreased uptake of serine (values above the ROC curve cut-off value) the PR's were 46.9 % compared to 13.5 % (OR 5.67, 95 % CI 1.97–16.32, $p=0.001$) (Fig. 1a). For zygotes with decreased uptake of proline the PR's were 43.2 % compared to 12.8 % (OR 5.21, 95 % CI 1.78–15.26, $p=0.003$) (Fig. 1b). For zygotes with decreased uptake of aspartic acid, PR's were 40 % compared to 18.5 % (OR 2.9, 95%CI 1.1–8, $p=0.04$) (Fig. 1c). No

significant differences in PRs were found in regard to zygotes' taurine uptake status (Fig. 1d).

To address the issue of double embryo transfers, we stratified the results of each amino acid according to the number of embryos that were transferred above or below the ROC curve cut-off value. Separately, we calculated the pregnancy rate for each subgroup. P values were calculated using linear by linear association. Additionally, the correlation between embryo morphology and pregnancy rate was also calculated for comparison with the metabolomics parameters.

When 2 high quality (grade A embryo, defined as day 3 or day 2 embryo which had ≥ 7 cells or four cells respectively without fragmentation) embryos were transferred the pregnancy rate was 38.5 %. The pregnancy rate for all other transfers (one high quality and one lower quality embryo, or both embryos of lower quality) was 33.3 % (NS). On day 3, 69.9 % of the embryos transferred were grade A, 26.5 % were

Fig. 1 ROC curves of amino acid metabolism on the first day after fertilization (day 1). **a** Serine, the nearest point to the optimum was -267.6016 , $p=0.003$. **b** Proline, the nearest point to optimum was

-4484.5 , $p=0.003$. **c** Aspartic acid, the nearest point to the optimum was -131.0081 , $p=0.04$. **d** Taurine, the nearest point to the optimum could not be determined

grade B and 3.6 % were grade C. No correlation was found between the zygote or embryo amino acid metabolism and embryo morphology or number of cells observed.

When both zygotes secreted above the ROC curve cut-off value of serine the PR's were 62.5, 12.5 % when both were below and 33.3 % when one was above and the other below ($p=0.04$). When both zygotes were above the cut-off value of proline the PR's were 66.7, 18.7 % when both were below and 28.6 % when one was above and the other below ($p=0.08$). For day 1 aspartic acid, the PR was higher in correlation with the number of transferred embryos secreting aspartic acid above the cut-off value (66.7; 42.9; 22.2 %), but did not reach statistical significance (Table 1).

There was absolutely no correlation between the metabolism of any of the studied amino acids during the cleavage stage and pregnancy, as measured by LCMS/MS in day 3 spent medium samples. Changes in the concentration of none of the four studied amino acids on day 3 were found to be in association with pregnancy (Fig. 2a–d).

Discussion

In this study we analyzed spent culture media of oocytes and embryos to identify a correlation between amino acids turnover and pregnancy. We established a non-invasive LCMS/MS based model to detect changes of four amino acids supplemented in our predominantly used culture media. We found that serine, and possibly proline, decreased uptake from the fertilization medium was associated with pregnancy. This observation was limited to the fertilization stage, suggesting that embryo's quality and implantation potential can be predicted even immediately after fertilization, and that reliable metabolomic fertilization stage biomarkers can be discovered and possibly used to select for transfer the embryo/s with the highest implantation potential.

Metabolomics has been proposed as an assessment strategy on the quest for an optimal method for embryo selection. As recent studies suggest, evaluating the embryo's interaction with its environment can reveal the embryo with the highest potential for viability [18, 13, 19, 8, 20]. Metabolomic profiling of the cleavage stage culture media can be accomplished with different rapid and non-invasive analytical techniques. Metabolites, such as pyruvate, glucose and lactate, were initially tested followed by amino acids turnover and oxygen consumption. The early studies of Whitten and Biggers, focused on metabolites, such as glucose, lactate and pyruvate, and embryo development [21, 22]. Leese was the first to study glucose and pyruvate turnover in animal and human embryo culture media using non-invasive microfluorescence techniques [23]. Studies on pyruvate metabolism showed that increased uptake is correlated with embryo development, but the overall results were inconsistent [24–27, 11, 3, 28]. Glucose consumption was associated with blastocyst development [10]; however, other reports failed to demonstrate

this relationship [27, 11, 29]. Studies regarding oxygen as a metabolite in the oocytes' fertilization environment, found an association between oxygen consumption embryonic development and implantation [13].

Amino acids play many roles in embryonic development, such as providing precursors for biosynthetic processes, like protein synthesis [30]. As the glycolytic intermediates, amino acids turnover has been studied. Amino acid profiling of human cleavage stage embryo culture media was first performed in 2002 using HPLC (high performance liquid chromatography). Houghton et al. [15] and Brison et al. [14] reported an association between altered amino acids levels in the culture media on day 2–3 and reproductive outcome [14, 15]. Houghton et al. reported that decreased uptake of glutamine, arginine and methionine on day 2–3 and serine on morula stage, and decreased production of alanine and asparagine on day 2–3, and alanine and glycine on morula stage, were associated with blastocyst development [15]. Brison et al. found that the turnover of leucine, asparagine and glycine was significantly correlated with clinical pregnancy and live birth [14]. Additionally, post-thaw amino acid metabolism was capable of predicting future development to the blastocyst stage [31]. Besides the importance of AAs profiling in predicting embryo's viability, a positive correlation between amino acids turnover and DNA damage was discovered [6]. Additionally, embryos that lost blastomeres during the cryopreservation and thawing processes had higher amino acids turnover [5]. These findings might be related to the Leese's Quiet Embryo hypothesis [32, 33] that postulates that metabolically quiet embryos with low metabolic rate are under reduced stress and have less need for genome repairs, hence they are more viable and possess better developmental competence. More recently, various studies have offered different models using optical and non-optical spectroscopic approaches for obtaining metabolomics data. There are reports of using NMR [34, 18, 35], NIR (near infrared regions) and Raman spectroscopy and Mass spectrometry [20, 36] for culture media metabolomics profiling. NIR and Raman spectroscopy have also been successfully employed to detect culture media metabolites. Seli et al. first reported the existence of a correlation between pregnancy and metabolomics profiling of the culture media combining NIR with Raman spectroscopy [37]. Other studies using NIR and Raman spectroscopy generated an algorithm of embryonic viability scoring system based on wavelet regions predictive with pregnancy [38–40, 3, 37, 4, 19].

Mass spectrometry (MS) based metabolomics has the ability to detect metabolites at the micromolar concentration level [41]. MS ionizes metabolites, separates them according to their mass to charge ratio, and then detects the separated ions. Additional information is provided with coupling MS with chromatography which separates molecules according to affinity and polarity. Separation techniques reduce the complexity of the mass spectrum. Liquid chromatography (the separation technique in this study) is known to simplify sample preparation since it can function in any temperature and does

Fig. 2 ROC curves of amino acid metabolism during cleavage (day 3). **a** Serine. **b** Proline. **c** Aspartic acid. **d** Taurine. There was no correlation between amino acid metabolism and pregnancy, therefore impossible to determine the nearest point to optimum for any of the studied amino acids

not require sample volatility [16]. The MS technology has been successfully applied in a variety of biological fluids and tissues and has been used not only on metabolomics, but also on proteomics in the field of ART [42, 43]. In regard to MS applied on embryo culture media, Marhuenda-Egea et al. evaluated retrospectively 25 samples after day 3 embryo/s transfer using liquid chromatography coupled to mass spectrometry [36]. They used a classification algorithm based on principal component analysis model, and showed differences

in relative amino acids concentrations in the culture media from embryos with differing implantation potential [36]. Cortezzi et al. also examined 113 embryos' culture media using mass spectrometry [20]. They classified the observed groups using partial least squares discriminant analysis. The detection and also abundance of specific ions (of amino acids and fatty acids) were observed and a MS-fingerprinting that distinguishes between groups (negative and positive implantation) was determined [20].

In the present study, we used liquid chromatography coupled with tandem mass spectrometry to detect alteration in four amino acid concentrations that exist in the most frequently used culture media. This combination provides the most current, accurate method to determine the change of metabolite concentration in the culture medium. Since the concentration of the studied amino acids in the culture media is subject to timely alterations during storage and culture, we provided for each case a control droplet from the same bottle cultured in parallel in the same incubator. We classified the transferred embryos according to their uptake or excretion status during fertilization and cleavage stages, and studied the correlation to their reproductive outcome. Thus, we were able to define specific biomarkers of implantation. In contrast to most published studies, we identified amino acids turnover during oocyte fertilization, rather than cleavage stage, as biomarkers of implantation. It is possible that part of the changes observed were oocyte related, as has been showed in other studies that the oocyte induce detectable changes in the culture media [44, 45, 9]. Therefore, it might be plausible that zygotes with a higher reproductive potential that require less amino acids supplementation from their environment develop into embryos more likely to implant. Serine surplus within the oocyte might represent a higher quality, resulting in an embryo with a better chance to implant. Serine is a cardinal proteinogenic component due to its ability to undergo phosphorylation. Its deficiency might represent a somewhat defective intracellular protein structure, and its surplus the opposite.

Detecting the AAs changes in the media was readily accomplished in our study. We were able to analyze individual samples in approximately 8 min long LCMS/MS analysis after prior preparation that took approximately 10 min per sample. Developing a technique that is simple safe and rapid is crucial in the IVF arena. Unlike spectrometric methods that examine the spent medium globally using patented devices that did not prove themselves in practice, the method we present is generic and is easily reproducible in any LCMS device.

We found no association between zygote or embryo metabolism of the studied AAs, and embryo morphological cleavage stage grading. Since we retrospectively compared the LCMS/MS result with the cycle outcome data, generally high quality embryos were preferred for transfer (almost 70 %). Despite that, there was no correlation between embryo morphology and pregnancy, or the amino acid profile of any of the embryos (those transferred and those chosen for cryopreservation) and morphological grading. This finding is consistent with other reports that demonstrated superiority for metabolomics profiling on morphology based selection [46, 4]. Morphology is considered limited in selecting the best embryo with the greatest developmental competence [1, 2], and was unable to distinguish between genetically normal and abnormal embryos.

Ideally, a single embryo transfer is preferred in defining the reproductive potential of each embryo individually; however, we were able to reach biologically meaningful and statistically significant results in women who had two embryos transferred. In a referral IVF unit specializing in older women, repeated implantation failures and PGD cases, elective single embryo transfers which meet this study's inclusion criteria are not common. In addition to the ROC curve calculations which define a threshold for the individual embryo, we calculated the pregnancy rate according to the number of embryos on each side of the ROC curve threshold. Significantly higher pregnancy rates were observed when both embryos transferred excreted serine and to a borderline significance proline, in comparison to transfers in which both embryos were below the threshold, or on both sides. We showed that metabolomic markers of reproductive potential can be identified very early after fertilization, and that information can be obtained within a time frame adequate for clinical diagnostic application.

Although the results are promising, a larger scale prospective, randomized study is required in order to define the applicability of our method. Since the results were insignificant for taurine and aspartic acid, future validation study will focus on serine and proline as day 1 biomarkers of implantation potential.

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Conflict of interest The authors declare that no commercial, financial or other conflict of interest exists.

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