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Metabolomics: Approaches to assessing oocyte and embryo quality

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Abstract

Morphological evaluation remains the primary method of embryo assessment during IVF cycles, but its modest predictive power and inherent inter- and intra-observer variability limits its value. Low-molecular weight metabolites represent the end products of cell regulatory processes and therefore reveal the response of biological systems to a variety of genetic, nutrient or environmental influences. It follows that the non-invasive quantification of oocyte and embryo metabolism, from the analyses of follicular fluid or culture media, may be a useful predictor of pregnancy outcome following embryo transfer, a potential supported by recent clinical studies working with specific classes of metabolites such as glycolytic intermediates and amino acids. Such selective approaches, however, whilst adhering closely to known cellular processes, may fail to harness the full potential of contemporary metabolomic methodologies, which can measure a wider spectrum of metabolites. However, an important technical drawback with many existing methodologies is the limited number of metabolites that can be determined by a single analytical platform. Vibrational spectroscopy methodologies such as Fourier transform infrared and near infrared spectroscopy may overcome these limitations by generating unique spectral signatures of functional groups and bonds, but their application in embryo quality assessment remains to be fully validated. Ultimately, a combination of evaluation criteria that include morphometry with metabolomics may provide the best predictive assessment of embryo viability.

Keywords: Metabolomics; Oocyte quality; Embryo quality; Metabolites; Mass spectroscopy

1. Introduction

At present morphological assessment is the primary method used to determine embryo viability during IVF cycles. It entails observations of the developmental pattern of embryos during culture such as the timing and rate of cleavage, fragmentation, inclusion bodies, cell number and allocation to the inner cell mass [1] and has the advantage of being a quick, convenient and inexpensive means of assessment but with modest predictive value [2,3]. Whereas the majority of human embryos are transferred during the early cleavage stages, the majority of embryos from domestic animal species are transferred at the morula or blastocyst stage. It follows that greater emphasis is placed on pronuclear morphology, cleavage timing and cell number in human embryos which tend to be handled in small numbers [2]. Embryo stage and overall morphology are the primary determinants in animal embryos where an internationally recognized system has been in practice for cattle since 1998 [3]. In general, however, morphological assessments are hampered by a lack of suitable standards and the inherent inter- and intra-observer variability associated with a subjective grading system. Furthermore, it does not necessarily equate to functional status, and morphologically normal looking embryos can still harbor genetic or epigenetic defects [4].

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Quantitative techniques have been developed for the non-invasive assessment of embryo metabolism and their value as predictors of embryo viability and pregnancy outcome is the subject of ongoing investigations [5–7]. The field is presently moving away from metabolic target analysis (analysis restricted to single or selectively defined metabolites) for diagnostic purposes towards metabolite profiling (which looks for selective groups of target compounds and their metabolic intermediates using a single analytical technique) and a more comprehensive analysis of the metabolome, which is the dynamic quantitative complement of all low molecular weight molecules (<1000 Da) present in cells at a particular physiological or developmental stage [8]. The hope is that the latter may enhance the value of embryo metabolism as a predictor of pregnancy outcome following transfer.

Low-molecular weight metabolites represent the end products of cell regulatory processes and therefore reveal the response of biological systems to a variety of genetic, nutrient or environmental influences. Since alterations to a cell's physiology, resulting from gene overexpression or deletion/silencing, are amplified through the hierarchy of the transcriptome and proteome, these changes can be more readily measured through the metabolome. In addition, the metabolome is downstream of gene function and therefore is a superior measure of cellular activities at the physiological level compared to trancriptomic and proteomic approaches. Increases in mRNA do not always correlate with increased protein expression [9] and a protein may or may not be enzymatically active [10]. It can be argued that changes in the transcriptome or proteome do not always correspond to altered cellular phenotype. This provides a further rationale for measuring the metabolome which could be an important complement to assessing gene function. The central dogma of molecular biology describes a linear unidirectional flow of information from gene to transcript to protein. Enzymes then regulate metabolic pathways and lead to phenotypic changes. This 'linear' flow of information is challenged by the fact that cellular phenomena are intimately networked with various feedback-loops and therefore should be represented as dynamic gene and protein complexes interacting with neighboring metabolites [11].

Metabolomics has, in recent years, proven to be a robust, high throughput and rapid post-genomic technology for pattern recognition analyses of biological samples [8]. Alterations to steady-state concentrations and transient changes in intracellular metabolites resulting from processes such as cell signaling can be readily investigated using metabolomic techniques such as gas chromatography–mass spectrometry (GC–MS), liquid chromatography–MS (LC–MS), capillary electrophoresis–MS (CE–MS) and nuclear magnetic resonance spectroscopy (NMRS), which are capable of detecting hundreds of individual chemical structures. Integrative analyses using genomic, proteomic and metabolomic techniques will help enhance the understanding of cellular physiology and lead to the identification of multiple physiological biomarkers that are not accessible with targeted studies. In this review we discuss current state-of-the-art metabolomic techniques and their applicability to the assessment of embryo quality and in developmental research.

2. Metabolomic analysis

The ultimate goal of a metabolomic experiment is to quantify all of the metabolites in a cellular system under a given set of physiological conditions at a particular time point. However, measuring the metabolome is a considerable analytical challenge. This is due to the labile nature, and wide dynamic and diverse range of many metabolites, their chemical complexity and heterogeneity, lack of simple automated analytical techniques which can quantitatively measure a large number of metabolites of unknown structure, the throughput of measurements and the elaborate nature of extraction protocols [12]. All these challenges need to be addressed by the analytical platform employed. Unlike transcriptome analysis, methods to amplify metabolites and therefore increase sensitivity do not exist. Furthermore, in contrast to genomics and proteomics, where only one class of compound is analyzed, metabolomic-based analyses have to deal with a diverse class of molecules with different properties.

Cellular metabolism has to be stopped as quickly as possible in order to terminate the activities of enzymes. Tissue samples should be snap-frozen to abruptly terminate metabolism. The metabolites then can be isolated by cold methanol or boiling ethanol, cold methanol:chloroform buffer, organic solvents, perchloric acid, or alkali. The advantages and disadvantages of these procedures along with their applications have been reviewed elsewhere [13].

The platforms for metabolome measurement should be unbiased, sensitive and have a capacity for high-throughput analysis to screen a large number of metabolites. Despite the enormous success in development of metabolomic technologies, it is not yet possible

Table 1
Contemporary metabolomic platforms and their applications

Metabolomic platform	Application and selectivity	Sensitivity (M)	Examples of metabolite analysis	Comments
NMRS	Little chemical bias and negligible sample preparation. Magic angle spinning NMRS can analyse small pieces of intact tissues. Useful for tissue extracts and biofluids	10^{-6}	Carbohydrates, oligosaccharides, amino acids, glycolytic intermediates, carboxylic acids	Provides detailed structural information. Low sensitivity reduces selectivity. Matrix effects can limit identification
LC–UV	Suitable for identification and quantification of a broad range of metabolites. No derivatization required. Suitable for thermo-labile compounds	10^{-9}	Glycolytic intermediates, carboxylic acids, amino acids, nucleotides, isoprenoids	Less suitable for identification unless coupled to MS
GC–MS	Broad selectivity, suitable for volatile and non-volatile metabolite separation, identification and quantification. Non-volatile compounds require derivatization, so can be difficult to identify. Not suitable for thermo-labile compounds	10^{-12}	Fatty acids, lipids in general, glycolytic metabolites, carboxylic acids, amino acids	Good separation but moderate throughput and solvent bias. GC on its own is less useful for identification unless coupled to MS
LC–MS	Suitable for identification and quantification of a broad range of metabolites. Derivatization prior to analysis not normally needed. Suitable for thermo-labile compounds	10^{-15}	Glycolytic intermediates, carboxylic acids, amino acids, nucleotides, isoprenoids	More suitable for polar compounds (solvent bias). But is also used for lipids. Matrix effects can limit identification. Moderate throughput
MS	Direct injection MS (DIMS) suitable for identification of a broad range of metabolites. Organic solvent extraction leads to loss of some compounds	10^{-15}	Carbohydrates, amino acids	Rapid throughput, but poor quantification. Metabolite identification of unknown compounds requires MS–MS. Matrix effects can be problematical
LC-LIF	Suitable for small metabolites since it has a mass detection limit between 10^{-18} to 10^{-20}	10^{-19}	Peptides, amino acids, catecholamines	Good for applications where the compounds have native fluorescence or can be derivatized
CE-LIF	Suitable for small (10^{-18} to 10^{-20}) polar metabolites using very small volumes. Provides high resolution but has low selectivity	10^{-23}	Amino acids, catecholamines, saccharides	As above or where the concentration and/or sample volumes are very small (volume = nL, concentration = 10^{-8} M)
FT-IR	Used to obtain metabolic fingerprints for a variety of biofluids including follicular fluid. Requires no reagents or sample preparation	10^{-6}	Vibrations associated with bonds and functional groups (e.g. C=O, N–H, C–N), amino acids, fatty acids, saccharides	Useful for identification of functional groups. Very high throughput. Little chemical bias. Direct use but requires sample dehydration

to analyze the diverse range of complex metabolites with a single analytical platform. The techniques most commonly employed for metabolomic analysis are NMRS and MS which can be used alone or preceded by a chromatographic step (Table 1). The data acquired through these platforms generate a large number of complex spectral signals, which reflect the metabolic status of the cell or organism. These large data sets require powerful statistical and bioinformatic tools for adequate data analysis and interpretation.

2.1. Nuclear magnetic resonance (NMRS) techniques

Various NMRS techniques have been used successfully to analyze the metabolome in biological samples. The use of this technique has been pioneered by Nicholson and colleagues [14,15] who used ^{1}H NMRS to analyze bio-fluids in order to diagnose disease. Although not as sensitive as MS, it is potentially fully quantitative using a single internal standard, requires little or no sample preparation, is highly reproducible and is capable of analyzing liquid and solid samples directly. NMRS can also detect isotopes of many elements such as ^{13}C, ^{31}P, ^{15}N and can therefore be used for examining metabolic pathways following exposure of biological systems to labeled low molecular substrates [12]. NMRS can be used also to investigate metabolic changes in intact tissues using magic angle spinning techniques [16]. The major disadvantage of NMRS is its relatively low sensitivity, which necessitates larger volumes. The issue of sensitivity has been addressed partly by improved detectors and development of magnets with increased field strength [17]. Whilst limiting the utility of this technique for direct, non-invasive measurements in the egg or pre-implantation embryo, the issue of sensitivity may be of less concern for analysis of metabolites in associated follicular fluid, granulosa cells or culture media.

2.2. Mass spectrometry techniques

Mass spectrometry (MS) is an extremely powerful analytical metabolomic tool since it provides a unique blend of sensitive, rapid, qualitative and potentially quantitative analysis of a large population of metabolites [13]. MS can be used alone or in combination with other chromatographic techniques. Without chromatographic separation, direct injection mass spectrometry (DIMS) involves the injection or continuous infusion of a crude sample extract straight into an electrospray mass spectrometer, an approach suitable for quick screening but not particularly quantitative [13]. Mass spectrometric analysis requires extensive sample preparation and extraction of samples usually with organic solvents which may lead to loss of certain compounds. Variable ionization efficiencies of metabolites hamper absolute quantification although concentrations of metabolites can be determined precisely by MS using stable-isotope internal standards [12].

MS can be combined with chromatographic techniques such as liquid chromatography (analytes separated by their chemical properties such as hydrophobicity), gas chromatography (analytes separated by their boiling point and interaction with the liquid layer covering the capillary in the gas phase), capillary electrophoresis (analytes separated by their mobility in a capillary filled with electrolyte under the influence of an electric field) or ultra performance liquid chromatography (UPLC). Recently developed UPLC systems use columns with very small particle size (<2 μm), which leads to almost a doubling of peak capacity and offers the highest efficiency attainable at relatively high linear velocities. These result in a 10-fold increase in speed and a three- to five-fold increase in sensitivity compared to conventional reversed phase high performance liquid chromatography (HPLC) [18]. LC–MS and UPLC–MS can be used for separation, identification and quantification of a broad array of metabolites and these techniques are highly sensitive, require no derivatization and enable analyses of thermolabile compounds (Table 1). The chromatographic step reduces the number of competing analytes entering the mass spectrometer and alleviates ion suppression and matrix effects in addition to facilitating separation of complex mixtures of metabolites, leading to improved MS data quality [19]. Capillary electrophoresis MS is a promising technology particularly suitable for the analysis of polar and thermolabile compounds, using very small volumes. GC–MS and LC–MS have moderate sample throughput but provide incontrovertible identification and quantification of individual compounds in a complex mixture. GC–MS is an extremely popular metabolomic platform due to its comprehensive nature and exceptionally high sensitivity. However, sample preparation is extensive, non-volatile metabolites require derivatization prior to analysis and thermolabile metabolites are essentially missed. Mass spectral deconvolution is needed to quantify metabolites that are not resolved by GC. Modern GC–TOF–MS applications are capable of faster spectral acquisition, and integrated deconvolution algorithms successfully accomplish these requirements [20]. In addition, Fourier transform–ion cyclotron

resonance (FT–ICR)–MS analysis is becoming increasingly popular since it is highly sensitive, and with its high mass resolution coupled to software that can use the information in isotope patterns, it can generate empirical formulae of metabolites directly [21]. The ability of FT–ICR–MS to generate spectral data relating to the elemental composition of metabolites suggests that this technique is set to play a major role in future metabolomic research.

2.3. Vibrational spectroscopy

Fourier transform infrared spectroscopy (FT-IR) is a high-throughput, non-invasive technique, which requires no reagents or sample preparation. It measures the vibrations of bonds within functional groups [22]. When a sample is subjected to light or electromagnetic radiation, chemical bonds absorb this light and exhibit stretching or bending vibrations, which can be, correlated to single bonds or functional groups of molecules for identification of unknown compounds. A large number of investigations have used FT-IR to analyze cells, tissues and biofluids for rapid detection of disease or malfunction [22,23]. Although most of the metabolomic investigations, which use vibrational spectroscopy have employed FT-IR, the use of Raman which, unlike FT-IR mainly measures non-polar bonds, is an emerging technology which has been used to assess embryo quality (see later). Near Infrared (NIR) spectroscopy, which primarily measures overtones and combination vibrations, also has significant analytical potential and has been used in various metabolomic investigations including measurement of lactate in human blood [22].

3. Metabolomic assessment of oocyte and embryo quality

Most of the relevant investigations into the assessment of oocyte and embryo viability have, to date, used targeted metabolic approaches [24] or have undertaken profiling for a selective class of metabolites such as amino acids or fatty acids using conventional chromatographic techniques [25,26]. The emphasis has been on hypothesis driven research, which has given rise to much of our current understanding of the metabolic requirements of mammalian gametes and the pre-implantation embryo [27]. This has since given way, at least to a certain extent, to metabolomic investigations that attempt to predict embryo quality. The profiling of one particular class of metabolite (i.e. amino acids) by HPLC has attracted greatest attention, and has been proposed as a means of non-invasive selection of developmentally competent embryos for transfer [5,6].

A recent retrospective clinical study employed this non-invasive method of embryo selection, which is based on the depletion/appearance of amino acids in culture medium [28]. The study involved 53 cycles of IVF treatment using ICSI. HPLC analysis of the spent medium showed that the turnover of three amino acids, asparagine, glycine and leucine, was significantly correlated with clinical pregnancy and live birth. Importantly, this association was found to be largely independent of other known determinants of pregnancy such as maternal age, ovarian reserve, embryo cell number and morphological grading, so that a combination of assessments (e.g. cell number, morphological grade and amino acid metabolism) could potentially increase the chances of selecting developmentally competent embryos. Allowing for differences in the composition of embryo culture media between studies, there appears to be great similarity in the metabolism of amino acids between pre-implantation embryos of contrasting mammalian species [26,28,29], indicating that non-invasive metabolic foot-printing of amino acid turnover has considerable generic potential to improve significantly the prospective selection of viable embryos for transfer.

Another study investigated fatty acid metabolism and uptake in donated human pre-implantation embryos by gas chromatography and showed that, compared to embryos which did not develop beyond the 4-cell stage, those that did develop beyond this point had significantly greater concentrations of the unsaturated fatty acids (particularly linoleic and oleic acids) and a lower concentration of saturated fatty acids [30]. Consequently, knowledge of the fatty acid requirements of the pre-implantation embryo could be used to enhance the success of IVF treatment. Non-invasive foot-printing of glycolytic activity has also been used for assessing embryo viability in mouse embryos [7]. Blastocysts with low glycolytic activity (i.e. low lactate production), in a range closer to that of *in vivo* derived embryos, were more viable than those with an abnormally high glycolytic rate. The use of glycolysis as an index for selecting viable embryos led to a four-fold increase in pregnancy rate compared to the transfer of embryos selected at random [7]. Metabolite profiling for glycolytic intermediates, therefore, offers another possible criterion for the assessment of embryo viability.

Less specific approaches than those listed above have employed techniques such as FT-IR to obtain 'biochemical fingerprints' for the composition of follicular

fluids from large and small antral luteinized follicles [23]. Given the importance of oocyte quality in determining post-fertilization development [31], the analysis of follicular fluid at the point of egg recovery could yield valuable predictive information concerning subsequent embryo viability. The results of Thomas and colleagues [23] showed that the FT-IR spectra of fluids collected from large antral follicles were tightly clustered, indicating a similar biochemical profile, whereas fluids collected from small follicles were heterogenous reflecting differences in their maturational stage. Further investigations are required to relate the FT-IR spectra of follicular fluid to the developmental competence of oocytes.

Finally, a recent prospective multi-centre study has shown that non-invasive metabolomic profiling of human embryo culture media correlated with pregnancy outcome [32]. This investigation used both Raman and Near Infrared Spectroscopy (NIR) to analyze culture media collected after embryo transfer on day 3 following fertilization. Results from this study showed that spectral profiles describing differences in –CH, –NH and –OH concentrations exhibit discrete differences between the culture media of embryos that resulted in pregnancy and those that failed to establish a pregnancy. The ratio of –CH to R–OH content in the media is reflective of oxidative stress. Using wavelength selective genetic algorithms (Molecular Biometrics, LLC, Chester, NJ) with Raman, the authors found four spectral regions associated with these molecular species to be predictive of pregnancy establishment. Raman analysis resulted in a specificity of 80% and a sensitivity of 95% whereas NIR provided a specificity of 83% and sensitivity of 73%. A similar investigation using NMR, Raman and NIR, analyzed 228 embryo media, 72 follicular fluid (FF) and 133 seminal plasma samples and identified specific oxidative stress biomarkers of R–OH, CH, OH and NH groups using wavelength selective genetic algorithms [33]. Results from this study showed that unique metabolomic oxidative stress profiles were consistently produced from discarded culture media, follicular fluid and seminal plasma and these profiles correlated well with pregnancy versus non-pregnancy outcomes.

4. Conclusions and perspectives

Metabolic profiling of follicular fluid or culture media can usefully serve as a high throughput and non-invasive means for the assessment of gamete and embryo viability, which could lower the number of embryos transferred during human ART procedures. This would lower the incidence of multiple births, and lead to a general improvement in pregnancy outcomes across species. Larger prospective studies, however, will be required to further refine and validate these methodologies in order to fully determine their value as predictors of egg or embryo quality. An important shortcoming with many of these techniques is the limited number of metabolites that can be determined by a single analytical platform under a given set of conditions. That is, the predictive value of metabolomic analysis may only be fully realized when the broadest range of metabolites can be determined. Vibrational spectroscopy methodologies such as FT-IR and NIR may overcome these limitations but their application remains to be fully validated. Ultimately, a combination of assessment criteria that includes morphometry with metabolomic analysis may provide the best predictive assessments of embryo viability.

Finally, two further considerations. First, the focus of current research endeavors has been directed primarily towards determining metabolites in culture media with little attention given to metabolites in follicular fluid or granulosa cells which are also retrieved at egg recovery. Could the metabolomic analysis of these superfluous materials provide additional predictive information on egg and embryo quality? Secondly, it remains to be determined if metabolomic analysis could indicate epigenetic defects, which may not affect embryo development and pregnancy establishment, but which could influence fetal development and the health and wellbeing of offspring.

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