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## REVIEW

# Non-invasive assessment of in-vitro embryo quality to improve transfer success




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**Abstract** Although IVF has been performed routinely for many years to help couples with fertility problems and in relation to modern breeding of farm animals, pregnancy rates after transfer to a recipient have not improved during the last decade. Early prediction of the viability of in-vitro developed embryos before the transfer to a recipient still remains challenging. Presently, the predominant non-invasive technique for selecting viable embryos is based on morphology, where parameters such as rates of cleavage and blastocyst formation as well as developmental kinetics are evaluated mostly subjectively. The simple morphological approach is, however, inadequate for the prediction of embryo quality, and several studies have focused on developing new non-invasive methods using molecular approaches based particularly on proteomics, metabolomics and most recently small non-coding RNA, including microRNA. This review outlines the potential of several non-invasive in-vitro methods based on analysis of spent embryo culture medium. 

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**KEYWORDS:** assisted reproductive technology, embryo quality, metabolomics, non-invasive assessment, proteomics, small non-coding RNA

## Introduction

For decades, in-vitro development of mammalian embryos has been performed extensively under laboratory conditions, and the method of choice to assess embryo quality has been based

on microscopic observations of morphology. Using this method, numerous morphological features related to embryo quality have been described, such as cell cycle timing, compaction times and cell shape (Holm et al., 1998 [bovine embryos]; Ziebe et al., 1997). Traditionally, morphological evaluation

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of embryo quality has been based on single-time observations; however, since the early 1980s time-lapse systems have become available (Massip and Mulnard, 1980). This allowed for very frequent morphological monitoring, which then has become a new addition to the range of methods for direct morphological assessment of embryo quality. During the last 10 years further refinements of morphological features monitoring have been introduced and semi-automated in a number of different set-ups (Holm et al., 2002 [bovine embryos]; Pribenszky et al., 2010; Rubio et al., 2014). Still, live birth rates using assisted reproductive technologies (ART) have not increased much over recent years (Kupka et al., 2014).

The challenge is to develop predictive methods that balance the wish to keep the embryo's viability unaffected by the observation with the need to obtain very detailed information to evaluate embryo quality with selection for transfer as the purpose.

There is a need to find other ways of additional or complementary evaluations, and different approaches have been suggested, mostly reflecting the embryo's function or physiological state. Studies have also been made to analyse the oocyte's surroundings, i.e. its cumulus cells or culture medium, but these methods have shortcomings and it has not yet been possible to apply any one of those to routine clinical settings. This review will therefore focus on outlining the potential for implementation into a clinical setting of various indirect and non-invasive in-vitro methods based on analysis of the spent culture medium of the embryo, within the areas of proteomics, metabolomics and small non-coding RNAs. One important consideration for the practical applicability of any method is its speed, in order to provide results within the time frame of the embryo culture.

### Indirect methods based on spent embryo culture medium

In spent embryo culture medium, the substances of interest can be divided into three main groups representing three different steps of cellular function: (i) the proteins translated from the specific gene expression products of the cells (proteomics); (ii) the end-products of the biological processes of the cells (metabolomics); and (iii) RNA-based negative regulators of gene expression (small non-coding RNA), which have been shown to be stable extracellularly (Jung et al., 2010). The analysis of these molecules is challenging as they are only present in limited amounts in the culture medium available for analysis, and thus methods need to be highly sensitive. The volume of the droplet of culture medium surrounding the embryo ranges from 15–40  $\mu$ l in single embryo cultures to 400–500  $\mu$ l in group cultures. However, although group culture results in higher amounts of analytes in the spent medium, this method does not give the ability to distinguish between individual embryos. Thus, sensitivity is imperative for any method to be considered relevant for spent embryo medium analysis.

### Proteomics

The proteome represents all proteins translated from the specific gene expression products of a cell at a specific time and

condition, whereas the embryonic secretome refers to the proteins produced and secreted by the developing embryo. The secretome profile of embryos is expected to change according to the viability of the embryo (Katz-Jaffe and Gardner, 2008), but unfortunately knowledge of the embryonic proteome and secretome is very limited. This, combined with constraints of proteomic technology, has been highly limiting for the development and application of proteomics for characterization of embryos. However, new technological advances, including increased platform sensitivity, have allowed discovery of potential biomarkers (Katz-Jaffe and McReynolds, 2013). These advances are still impeded by technical problems associated with the reagents used for embryo cultures, in particular because many laboratories use serum containing albumin as a protein source in the culture medium. It has not yet been possible to find a replacement for albumin, as the growth of the embryos has been shown to be affected by alternatives to albumin (Mains et al., 2011). The presence of albumin in the serum makes it very difficult to detect proteins with a molecular weight corresponding to that of serum albumin (60–70 kDa) using methods that are cost-efficient and applicable to IVF laboratories (Mains et al., 2011).

Several attempts have been made to establish consistent protein biomarkers in embryonic medium. In 2009 Katz-Jaffe et al. reviewed findings on potential protein biomarkers in the embryo secretome (Katz-Jaffe et al., 2009), including platelet-activating factor, leptin, agrocraanin, human leukocyte antigen G (HLA-G) and ubiquitin. Although these all appear to be promising biomarkers for prediction of transfer success, only HLA-G has been investigated in a clinical setting for its ability to predict pregnancy outcome. HLA-G has strong immunosuppressive functions and is thought to be one of the most important factors in reshaping the maternal immune response into tolerating the fetus (Jurisicova et al., 1996). However, in spite of several promising proof-of-concept studies (Desai et al., 2006; Fisch et al., 2007; Fuzzi et al., 2002; Noci et al., 2005; Rebmann et al., 2007; Shaikly et al., 2008; Sher et al., 2004, 2005a, 2005b; Yie et al., 2005), multi-centre trials have found that HLA-G does not offer any clinical advantages compared with morphological scoring, which seems to be due to variation in HLA-G contents in embryonic media between different ART centres (Rebmann et al., 2010; Tabiasco et al., 2009).

More recently, the focus has been on highly multiplexed proteomic methods, such as mass spectrometry (MS). Using such methods Cortezzi et al. (2011) identified 25 proteins in the secretome, of which 15 predicted positive pregnancy outcome and 10 were associated with negative pregnancy outcome. The Jumonji protein (JARID2) was the most represented of the proteins that predicted positive pregnancy outcome. JARID2 is part of a histone methyltransferase complex that is involved in the regulation of embryonic patterning genes (Pasini et al., 2010). However, none of these proteins were investigated as clinical biomarker in randomized controlled trials. MS was also used to establish that the concentration of apolipoprotein A1 (ApoA1) in spent embryo medium was higher at culture day 4–5 of embryos of higher morphological grade compared with abnormal embryos (Mains et al., 2011); however, the concentration of ApoA1 in the spent medium was not correlated with pregnancy outcomes. ApoA1 is associated with high-density lipoprotein (HDL) (Santos-Gallego et al., 2008) and plays a key role in

cholesterol transport (Brunham et al., 2006). This study was elaborated in Nyalwidhe et al. (2013) focusing on the secretome of day 2–3 embryos using MS, in which ApoA1 was also found to be highly represented; however, in this study ApoA1 was less abundant in the samples associated with successful pregnancy compared with those that were not. The authors confirmed this using enzyme-linked immunosorbent assay (ELISA) and western blotting on pooled media samples. Based on these results and earlier data the authors suggested that ApoA1 internalization could be characteristic of viable embryos leading to a correlation between successful transfer and low concentrations of ApoA1 in the secretome early in development. This was further substantiated by the finding of ApoA1 mRNA transcripts in day 5 blastocysts but not in day 3 embryos (Mains et al., 2011), indicating a higher intracellular concentration of ApoA1 in the blastocyst compared with the embryos. In a recent study of the proteome of bovine embryos (Deutsch et al., 2014), proteins involved in lipid metabolism decreased during embryo development, and in particular ApoA1 abundance decreased by 1.6-fold from the oocyte stage to the 4-cell stage. The authors speculated that this decrease might be caused by an increased secretion by the embryo into the culture medium during embryogenesis. Unfortunately, in this study there was no discrimination between viable and non-viable embryos, nor an investigation into the secretomic profile of ApoA1 to support the human data of differences in the ApoA1 abundance in the spent embryo medium.

In another approach, Butler et al. (2013) used MS to investigate the hormone human chorionic gonadotrophin (HCG) in the secretome. As a signal for maternal recognition of pregnancy, HCG has long been used as an obstetric marker for embryonic development (Cole, 2009). The authors speculated that different isoforms of HCG could predict the transfer success of the embryo. Out of four isoforms, HCG $\beta$  appeared to have potential as a biomarker as it was detectable in the spent medium as early as at the 2-cell stage in some samples using both MS and immunoassays, along with concurrent gene expression. It was not possible to detect HCG in the spent medium prior to embryo hatching (on day 6 for the human embryo). Furthermore, it seemed that the presence of the HCG $\alpha$  isoform early in development might be an indicator of pathology, as it was detected in the culture medium of abnormal (3-pronuclei) embryos. However, this study did not correlate the data to pregnancy outcomes, only to embryo morphology.

Although there is a lot of activity in the area of discovering secretome biomarkers, a number of huge obstacles must be overcome before early, sensitive and consistent protein biomarkers of embryo quality can be found. Apart from the restrictions already mentioned (small sample volumes, low analyte concentrations), other practical and ethical concerns make it difficult to perform detailed assessments of potential biomarkers in humans; for example, single embryo transfers are not a realistic approach in most fertility laboratories, which makes the determination of the accurate pregnancy rates a problem. Furthermore, the data are confounded by the fact that most proteome studies are performed on pooled samples, making it difficult to distinguish between different embryos outcomes. In order to increase the utility of proteomics for prediction of success of embryo transfer, further development of proteomic technologies is therefore

needed so that single samples and even lower protein concentrations can be measured. Furthermore, the increased possibility to design proper experiments in model animal embryo cultures could be pursued even more and more basic investigations in appropriate animal models will be needed.

Mass spectrometry-based proteomics methods have the advantage of speed compared with traditional immunochemical methods such as ELISA and therefore possess a greater potential for application to ART.

## Metabolomics

Metabolomics provides a snapshot of the concentrations of all metabolites in the culture medium as detected by the applied method. Metabolites can change according to the metabolic or environmental state of a cell. The metabolome (small molecule metabolites found within a biological sample) therefore is a good indicator of cellular activity, and may present good biomarkers for viable embryos having a high probability of success after transfer. In contrast to genomics and proteomics, metabolomics deals with diverse classes of molecules, such as amino acids, oxidation products, carbohydrates and carboxylic acids (Nagy et al., 2008).

The most intensively studied class of non-invasive biomarkers is the secreted metabolome, and several promising metabolite biomarkers have been discovered (the most recent are shown in Table 1 and further are reviewed in Botros et al., 2008 and Nel-Themaat and Nagy, 2011). Until recently, most efforts have used spectroscopy, especially near infrared (NIR) spectroscopy, for investigating possible links between the metabolome and embryo viability. This technology measures vibrations of functional groups in a sample without identifying specific metabolites, leading to the generation of a viability score by comparing regions within the NIR spectral profiles (Botros et al., 2008). The purpose is to derive a unified measure discriminating between viable and non-viable embryos (Ahlström et al., 2011). The advantages of NIR include that measurements can be made directly on even small sample volumes (<15  $\mu$ l), with no requirements for sample preparation and with results being available within 1 minute per sample (Seli et al., 2007). This is in contrast to other spectroscopic platforms, such as Raman and nuclear magnetic resonance (NMR) spectroscopy, characterized by expensive and complicated equipment, low sensitivity, lengthy and extensive sample processing, or the need for complex statistical analysis for interpretation of results making them impractical in clinical settings (Rinaudo et al., 2012; Seli et al., 2010).

Several studies have focused on NIR as a potential way to assess embryo viability, whether used alone or in conjunction with morphology assessments, and results have been promising (Ahlström et al., 2011; Fu et al., 2013; Nagy et al., 2008, 2009; Seli et al., 2010, 2011; Vergouw et al., 2008, 2011). However, randomized controlled clinical trials have generally shown that NIR-based metabolomics profiling did not offer any advantage over morphologic assessment of embryos. In a meta-analysis of four such studies, the live birth rates were 34.7% in the control group and 33.2% in the NIR group, which was not a significant difference (Vergouw et al., 2014). A problem in most NIR studies is a large intra-group variation, which can lead to both false positive and false negative

**Table 1** Overview of recent metabolomic findings.

Study	Method	Species	Day	Sample size	Findings
Wiener-Megnazi et al., 2011	TLC	Human	2 and 3	133 patients–284 embryos	Oxidative status can be used as a transfer marker at day 3
Vergouw et al., 2011	NIR	Human	5	115 patients–127 embryos	Correlation between increased viability score and live-birth outcome
Seli et al., 2011	NIR	Human	5	198 embryos	Viability score better indicator for transfer outcome than morphologic grading
Gardner et al., 2011	Micro-fluorimetry	Human	4 and 5	50 patients–50 embryos	Increased glucose uptake indicates higher pregnancy rate and female embryos have higher uptake than male
Combelles et al., 2012	ELISA	Human	3	91 patients–122 embryos	SOD1 (superoxide dismutase-1) cannot be used as a biomarker for transfer potential
Pudakalakatti et al., 2013	<sup>1</sup> H NMR	Human	2 and 3	48 patients–127 embryos	Pyruvate/alanine ratio can predict transfer outcome at day 3
Fu et al., 2013	NIR	Human	3	172 patients–239 embryos	Viability index predicts transfer outcomes
Wallace et al., 2014	<sup>1</sup> H NMR	Human	2	37 patients–58 embryos	The ratios between different metabolites are correlated with transfer potential
Kirkegaard et al., 2014	<sup>1</sup> H NMR	Human	3 and 5	161 patients–148 embryos	No correlation between NMR variations and pregnancy outcome
Vergouw et al., 2014	NIR	Human		924 patients	Meta-analysis of four studies: NIR profiling does not improve birth rates
Muñoz et al., 2014a	FTIR	Bovine	6 and 7	69 embryos	In a subgroup of blastocysts, using FTIR is correlated with higher birth rates
Muñoz et al., 2014b	FTIR	Bovine	7	49 embryos	FTIR profiling correlates with viability of in vivo embryos however is affected by embryo management procedures

ELISA = enzyme-linked immunosorbent assay; FTIR = Fourier transform infrared spectroscopy; <sup>1</sup>H NMR = proton nuclear magnetic resonance spectroscopy; NIR = near infrared spectroscopy; TLC = thermochemiluminescence.

predictions. In 2013 an NIR instrument prototype specifically developed for clinical practice in ART centres was withdrawn from the market (Molecular Biometrics, Norwood, MA, USA) (Uyar and Seli, 2014), and the present status is that the NIR technology needs to be improved and retested before new attempts to use it for assessment of embryo viability can be made, which will happen as we get more knowledge about the embryonic metabolomic profile.

Recent efforts have utilized other spectroscopy methods. One is a variant of NIR, namely Fourier transform infrared (FTIR) spectroscopy, which has been used to predict pregnancy viability of bovine embryos. The studies were performed with spent medium collected at day 7 from in-vivo (embryos recovered at day 6) and in-vitro produced embryos (Muñoz et al., 2014a, 2014b). Although the authors found that the metabolome of the spent culture medium was different between embryos that were successfully transferred (end-points were day 60 pregnancy and live birth) compared with those that were not, FTIR-based metabolomic profiling did not lead to higher specificity of prediction of transfer success compared with conventional embryo selection methods. However, when applied to a subgroup of in-vitro expanded blastocysts, which accounted for 66% of the embryos transferred in this study, the FTIR-based metabolomic profile of spent culture medium was correlated with a substantially higher birth rate than when the method was applied to embryos of all stages (82.8% versus 72.2%, respectively) (Muñoz et al., 2014a).

Furthermore, the authors found that differences in the treatment of embryos (fresh or vitrified) and between the laboratories that handled the embryos introduced differences in the FTIR profile of spent culture media (Muñoz et al., 2014a, 2014b). Although there is potential in using FTIR to predict transfer success, as the method is fast, easy to handle and inexpensive, it is limited by the relatively small amount of validated data in presently available databases (Muñoz et al., 2014a). Furthermore, the studies done so far using FTIR have not been blinded, which is a reason for caution.

Finally, a number of recent studies on metabolic profiling have used NMR spectroscopy, which is able to identify specific metabolites, unlike NIR (Rinaudo et al., 2012). Results, however, are ambiguous. Pudakalakatti et al., 2013 found that a low pyruvate/alanine ratio in embryo culture medium on day 3 (but not on day 2) was correlated with successful transfer, and Wallace et al. (2014) identified and quantified 12 metabolites in spent embryo medium, of which seven had not been investigated before in relation to prediction of pregnancy success. They found that the ratios between these metabolites on day 2 differed between embryos that were successfully transferred and those that were not, and therefore suggested the use of such ratios for predicting transfer success. However, other studies have found that NMR cannot be used to predict transfer success using multivariate analysis. For example, Rinaudo et al. (2012) investigated the NMR profile of day 3 spent embryo medium and found that

implanted and non-implanted embryos did not show consistently different NMR profiles. Interestingly, these authors compared the profiles of three different types of spent embryo media, and found that the medium composition profoundly affected the NMR profile. As different IVF laboratories use different types of culture media and as manufacturers continuously change and improve culture media, this presents a major problem with this method. So far, Kirkegaard et al. (2014) is the only study using NMR to only include spent embryo medium from single transferred embryos, making it possible to assess the transfer success of each embryo. They investigated spent embryo medium from day 3 and 5 embryos and found that in spite of a high degree of individual metabolomic variation, it could not be associated with pregnancy outcome. It is not clear why conclusions of these four studies are this different; however, one reason could be differences in the sample sizes. Both of the studies that did not find any correlation between NMR profiles and transfer success used larger sample sizes ( $n = 108$  and  $161$ , respectively) compared with the studies that did find a correlation ( $n = 48$  and  $37$ , respectively). These findings, coupled with the fact that NMR as a method is not readily applicable in a clinical setting, due to the cost and size of the equipment as well as the special expertise required (Rinaudo et al., 2012), the potential for using NMR as a practical clinical tool for predicting embryo transfer success remains low at present.

In conclusion, although the area of metabolomics has been heavily investigated as a way to predict embryo viability, and several possible biomarkers have been identified (summarized in Table 1), no single or composite early, consistent and sensitive biomarker for clinical use has been established. However, advances in the technology and the databases may change this in the future, and spectroscopic methods such as IR and NMR are fast and potentially readily applicable to embryo quality prediction in an ART setting.

### Small non-coding RNA

Recently, a new approach to non-invasive screening of spent embryo medium was suggested. It involves detecting microRNA (miRNA) directly in the spent embryo medium. The importance of small non-coding RNA, such as miRNA, for embryogenesis and development is clearly established (reviewed in Galliano and Pellicer, 2014) both in humans and in other species of interest, including pigs (Prather et al., 2009), cattle (Goossens et al., 2013) and chickens (Darnell et al., 2006). miRNA are small (18–22 nucleotides), non-coding RNA molecules that are mostly considered to be negative regulators of gene expression as they can destabilize or repress translation of messenger RNA (mRNA) (Bagga et al., 2005; Wu et al., 2006). One type of miRNA can potentially regulate more than a hundred different target genes (Lim et al., 2005). Interestingly, the presence of miRNA has been detected in almost all biofluids including tears, blood, urine, breast milk and semen (Weber et al., 2010), and in cell culture medium (for example Valadi et al., 2007). Furthermore, extracellular miRNA is very stable and resistant to degradation (Jung et al., 2010). This might be due to the encapsulation of at least some extracellular miRNA in small membrane-derived vesicles called exosomes, which can be

delivered to other cells and thereby impact the gene expression in those cells (Valadi et al., 2007).

In humans, the miRNA profile of blastocysts was found to be aberrant in blastocysts from groups of patients with either male factor infertility or polycystic ovaries compared with blastocysts from patients with normal fertility, even though the blastocysts had the same overall morphological quality (McCallie et al., 2010). In this study, the expression of 12 miRNAs was investigated in three blastocyst types, and clear differences in the miRNA profiles of the blastocysts from the infertile patients compared with blastocysts from patients with normal fertility were found.

In 2013, the group of Katz-Jaffe and McReynolds analysed spent blastocyst culture medium and failed to detect any of the 377 miRNAs in their analysis panel (Katz-Jaffe and McReynolds, 2013). Since then the sensitivity of methods for identifying miRNAs has greatly increased, leading to two studies published in 2014 claiming that embryos do indeed secrete miRNA into the culture medium, and that the miRNA profile could possibly be used as a biomarker to predict transfer success (Kropp et al., 2014 [bovine and human embryos]; Rosenbluth et al., 2014). Rosenbluth et al. (2014) studied 754 human miRNA and found that three (miR-372, miR-191 and miR-645) were differentially expressed on day 5 between the spent culture medium of embryos that had successful pregnancy outcomes and spent culture medium from embryos that did not lead to pregnancy. No correlation was found between the miRNA profile and pregnancy outcome in the spent culture medium of embryos produced by intracytoplasmic sperm injection (ICSI), or when the spent culture medium of IVF embryos and ICSI embryos were pooled. The miRNA concentrations were too low to be detectable at earlier time points. Interestingly, it was also discovered that miR-191 was found in higher concentrations in the spent culture medium of aneuploid embryos compared with euploid embryos. Kropp et al. (2014) studied five miRNAs in bovine and human embryos and in spent embryo medium of either embryos that developed into blastocysts or degenerate embryos (day 8 in cattle and day 6 in humans). They found three of the five miRNA in the medium (miR-25, miR-181a and miR-370), but with no difference in amounts or miRNA ratios between the two embryo types. However, miR-196a2 was only found in the medium of abnormal blastocysts and blank medium, leading to the authors suggesting that it was taken up by viable blastocysts. Interestingly, these authors found a difference in the miRNA gene expression of the blastocyst compared with the miRNA found in the medium, suggesting that embryos selectively secrete or take up certain miRNAs. Unfortunately, this investigation was hampered by the fact that the spent culture medium was pooled and that only five miRNAs were investigated. Furthermore, the authors did not investigate whether there was any correlation between pregnancy rates and miRNA profile.

The miRNA results are promising and indicate that the types and amounts of miRNA secreted into the culture medium might differ between viable and non-viable embryos. The general interest in using miRNA as disease biomarkers is growing rapidly, also speeding up the development of new and more sensitive methods for detection of miRNA in biofluids, leading to more generally applicable and easier to use methods. The quantitative real-time polymerase chain reaction (qPCR) method is fast, relatively cheap and can be

used with very small sample sizes. Although some sample pre-treatment is needed, this makes it a candidate as a tool to detect miRNA biomarkers in embryo culture medium as the test can be performed in-house on the spent embryo medium before transfer. However, it is clear that more investigations are needed into more types of miRNA, in larger experimental settings with more samples, and in different laboratories using different types of culture media, so hopefully it will be possible to identify an early, sensitive, distinctive miRNA profile to select the embryos having the best chance of pregnancy and birth.

## Conclusion

The area of non-invasive screening of embryo quality is a very active one and the focus remains on identifying non-invasive biomarker-based methods to supplement the traditional morphological assessment. In the field of metabolomics especially the methods using infrared spectroscopy (NIR and FTIR) might be candidates for clinical applications, as they are rapid and easy to handle, and the miRNA profiling of embryo medium using qPCR certainly has potential as well. Both qPCR and MS-based proteomics require some level of sample pre-treatment before analysis and also do demand some time for the analysis itself; however, this can still be fitted within the ART scheme. The main challenges with the use of these methods in a clinical setting remain low platform sensitivity, issues with the practical management of cultures and samples (for example, different types of embryo culture media used in different IVF laboratories or the use of double embryo transfer) and lack of knowledge of the biological processes in the developing embryos such as the limitations of the metabolomic databases and of regulation of miRNA.

Even so, several potential possible biomarkers have been proposed, especially in the field of metabolomics (see [Table 1](#)), although this has not so far resulted in higher pregnancy or birth rates, and at present no biomarker clearly stands out as a potential early, consistent and sensitive means of predicting transfer success. However, this will hopefully change within the next few years as technologies keep improving, and as new biological discoveries are made. Furthermore, more studies are utilizing animal models to increase the understanding of the complex system of embryogenesis in mammals. Until then, the use of morphological assessment of pre-transfer embryos continues to be the best tool for assessment of embryo quality.

## Declaration

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