Unconventional embryo selection strategies: a look into the future

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Summary

The assessment of an unequivocal method able to identify the embryo with the higher possibility to implant, leading to a single live birth, is crucial for the success of an infertility treatment. Standard morphology evaluation, morphokinetic analysis and preimplantation genetic screening are the more employed embryo selection strategies in reproductive medicine centers. However, the results and the applicability of these methodologies are still controversial. Therefore, there is the need to find additional technologies to clinically select developing embryos. The challenge is to set-up a predictive, not invasive, reproducible, objective and effective method able to select the best embryo. In this review, we present several unconventional methodologies of embryo evaluation, analyzing their possible future application in human reproduction.

KEY WORDS: embryo selection, embryo quality, mtDNA, metabolomic, oxygen consumption.

Introduction

A lot of intrinsic and extrinsic factors have been recognized having an influence on human embryo development and quality. The ovarian stimulation protocols, the methods of oocyte insemination, the choice of culture media, the culture conditions, the duration of in vitro culture and the timing of embryo transfer were all hypothesized to have an effect on biological and clinical outcomes (1-8). When an embryo is produced in vitro, there is the need to evaluate its developmental and implantation potentials. The ideal approach should be reliable, quick, simple, reproducible, objective, cheap, independent from other variables and should not impair the embryo itself. So far, two general types of embryo selection strategies are available: invasive and not invasive. In the second group, the standard morphological evaluation is the most longstanding and widespread technique for embryo quality evaluation. However, it has been extensively demonstrated that morphology has only a weak correlation with embryo’s capability to implant leading to a healthy live birth, especially when evaluated in the first stages of development (9, 10). At the end of the last century, it has been shown that blastocyst transfers allow improved clinical outcomes compared to previous stages (11, 12). Consequently, extended culture has been proposed as a way to select and identify the best embryo (13). Anyway in 2016, Gluvovsky and coauthors (3) reported that there is still low quality evidence that blastocyst stage is associated with higher clinical pregnancy and/or implantation rates than cleavage stage transfers. In addition, extended culture has also hypothesized to be responsible of epigenetic changes and blastocyst transfers have been linked to monozygotic twinning and perinatal complications (14). Recently, the time-lapse imaging has becomes available in order to evaluate the morphokinetics of embryo development. Quickly a lot of studies, reporting algorithms and models that may assist in selecting the most viable em-
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Specific gravidity system

A very innovative embryo selection strategy, based on a gravidity technique, was recently proposed by Prien et al. (21). In this preliminary animal-based in vitro study, the possibility to discern viable and nonviable embryos by mean of a specific gravidity system (SGS) was tested using a mouse model. The SGS method was developed on the basis of previous observations performed on cattle and mouse embryos, suggesting that embryo weight could be a good indicator of biochemical status influenced by maternal body composition, especially body fat (22). The SGS is composed by a column filled with culture media and positioned on a plate. The embryo is placed on the opened top of the chamber and the timing employed to pass through 1 cm, called timing zone, is measured. The speed of fall down depends from the object’s density and viscosity: comparing objects with similar shape and size, the speed is directly correlated to weight. In this way, Authors were able to determine embryo’s weight after building a standard curve obtained measuring the descent time in seconds of known weight beads. In the first experiment, 78 1-cell embryos from 5 mice were split in two groups. In the control group, 39 embryos were tested in SGS as previously described and placed in culture. In the study group, 39 embryo’s descent times were measured before and after killing them by mean of 60°C heating, and then they were placed in the same culture conditions as the control group. The descent rates were measured again every 24 hours for both groups. Control embryos showed increasing weights from 1-cell to morula stages and finally a decrease at blastocyst stage. The killed embryos demonstrated a highly statistical relevant deviation from the control pattern in the first 48 hours of culture, probably due to changes in membrane integrity. In the second experiment, 414 1-cell embryos from 27 mice were split in two groups: 207 of them were placed directly in culture and their morphology was evaluated daily; the remaining 207 embryos were previously passed through the SGS and then cultured and evaluated in the same way then the others. Growth rate of embryos checked with SGS was similar to embryos placed directly in culture and blastocyst formation rate was even higher after SGS than in control group. In addition, it has been found that among tested embryos, those that reached the blastocyst stage showed a pattern comparable with the U-shaped standard curve, while most of the embryos with the longest or shortest descent times degenerated during the culture. This series of experiments demonstrated that SGS could be a safe technique able to reveal differences in chemical constitution of zygotes morphologically identical, which would be expected to influence em-
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MicroRNA
Another pioneering embryo selection strategy could involve the characterization of extracellular microRNAs (miRNAs) secreted in spent culture media and their possible use as biomarkers (23, 24). Over 1,000 miRNAs have been identified in human genome: they are stable, noncoding, single-stranded RNA molecules and they are considered to be the major negative transcriptional regulators of gene expression. MicroRNAs are secreted in exosomes and microvesicles and can be detected in all body fluids. It has been hypothesized that miRNAs could play a role in the blastocyst-endometrium interaction during the implantation process (25). In human, aberrant miRNA profiles were described in blastocysts deriving from infertile patients with either male factor infertility or polycystic ovaries respect to blastocysts from patients with normal fertility, independently from their morphological grading (26). In 2014, two studies were published demonstrating that embryo secrete miRNAs into the culture medium and their possible employment as biomarker (27, 28). In the first study (27), the expression of five miRNAs in human and bovine embryos was analyzed. It was observed for the first time that miRNAs are expressed in spent culture media from both bovine and human embryos. In addition, it was highlighted a differential miRNA gene expression in bovine embryos that reached the blastocyst stage, compared to degenerated embryos, hypothesizing a possible correlation with developmental competence (27). In the second study (28), ten human miRNAs were identified in culture media and it was found that three of them (miR-372, miR-191 and miR-645) were more highly concentrated in spent culture media from failed in vitro fertilization (IVF) cycles. In addition, one of them (miR-191) was also found in higher concentration in culture media from aneuploid compared to euploid embryos (28). Later, in a prospective cohort study, the miRNAs in the spent culture media from 25 implanted and 28 not implanted blastocysts after 12 hours of culture after warming, obtained in 44 consecutive IVF cycle, were analyzed (24). First of all, it was found that the majority of miRNAs detected in more than three TE biopsies and/or spent culture media samples were shared among all samples examined. In addition, 57 out of 59 (96.6%) miRNAs detected in the spent blastocyst culture media were expressed also from the TE cells, suggesting that miRNAs in spent culture media could be released from TE. On the contrary, analysis of miRNAs in the media collected from cleavage and morula stages embryos was comparable to the negative controls, suggesting that miRNAs profiling is applicable only to blastocyst stage. Finally, the comparison of spent culture media from euploid implanted versus euploid not implanted blastocysts highlighted that in 96% of the implanted ones two miRNAs (miR-20a and miR-30c) were statistically differently expressed, showing increased concentrations in media from implanted blastocysts. It is interesting to note that these two miRNAs are involved in different pathways and biological processes mainly involved in cell-to-cell communication, signaling, adhesion and cell growth/cancer that could be all ascribable to a potential communication system during the implantation. Anyway the direct proof that endometrial cells internalize miRNAs is still to be proven. The potentiality of using miRNAs as biomarkers of implantation is of great interest in the field of human reproduction, due to the fact that it should be a not invasive, stable over the time, specific to embryos and easily measurable procedure. The development of new and more sensitive method for characterization of miRNAs in small volume of fluids will lead to more general applicability of this method (23). Nevertheless, this strategy need to be further explored, analyzing more types of miRNAs in larger experimental setting, before to being introduced in clinical routine. A prospective multicenter study is currently ongoing where a panel of 46 selected miRNAs will be used in order to confirm these preliminary findings on a higher and defined sample size (24).

Mitochondrial DNA
Mitochondria are involved in a lot of cellular processes, such as apoptosis, amino acid synthesis, calcium homeostasis and the generation of energy (29-31). These organelles contain some copies of circular, double stranded mitochondrial DNA (mtDNA) that are involved in cellular metabolism (32, 33). Mitochondria...
content in mammalian cells ranges from a few hundred to thousands, depending from cell volume and energy request. As a consequence, human mature oocyte has a very high content of mitochondria mtDNA (32, 33). In mammalian embryos mtDNA is only maternally inherited, deriving exclusively from the initial mitochondria population present in the oocyte before fertilization. The amount of mtDNA remains stable during the pre-compaction embryo’s development and it is thought that a significant replication starts only after the differentiation into TE and inner cell mass has initiated (30, 32-34).

In summary, mitochondrial functions seem to be critical during the first days of embryo development and for this reason it has been proposed that they could represent a good biomarker of embryo viability. In 2014, Stigliani and coauthors (35) investigated if the ratio between mtDNA and genomic DNA (gDNA) released in culture media by the embryos is correlated with embryo morphology, blastocyst development and implantation potential. The analysis was performed on day-3 spent culture media from 699 embryos. A significantly higher mtDNA/gDNA ratio was found in the culture media of embryos that successfully developed into blastocyst stage, compared to arrested embryos. In addition, good-quality embryos that reached the blastocyst stage on day-5, showed higher mtDNA/gDNA ratio in their spent culture media respect to those that developed slower. Finally, higher TE quality and successful implantation outcome was associated with higher mtDNA/gDNA ratio. The Authors concluded that combinating quantification of mtDNA/gDNA ratio with morphological evaluations could be a non-invasive early biomarker of embryo viability. In 2015, Fragouli et al. (33) analyzed the cellular embryo’s content of mtDNA and its relationship with female age, ploidy status, viability and implantation potential. A total of 39 cleavage stage embryos and 340 blastocysts were biopsied and citogenetically tested. The quantity of mtDNA resulted higher in embryos from older women as well as in aneuploid compared to euploid blastocysts, independently from female age. Finally, implanted blastocysts had generally lower mtDNA level compared to not implanted ones. Authors were able to establish an mtDNA quantity threshold, independent from blastocyst morphology and female age, above which implantation was never obtained. These data were confirmed by another study (37) in which the mtDNA content, from 205 blastomeres removed from day-3 euploid embryos and 65 trophoectoderm biopsy samples, was analyzed. It was found that the mtDNA level in day-3 embryos successfully implanted was significantly lower compared to those that failed to implant. Authors generated a mitochondrial score on the basis of the relative mtDNA content able to predict viability of euploid embryos. They found also a trend toward an increased mitochondrial score in poor quality embryos and confirmed that mtDNA content is not related to female age. These findings are supported by the observation that mitochondrial dysfunction is associated with mitochondrial hyperproliferation: the hypothesis is that an increase in mtDNA copy number in an early embryo could represent some metabolic stress (38). In contrast to these reports, a study based on the analysis of mtDNA content from 1,396 embryos showed no statistically significant differences in blastocysts grouped by ploidy, maternal age or implantation potential (39). The substantial innovation was the development of a mathematical formula taking into account the genomic variation deriving from the protein supplement of culture media.
from confounding factors, such as embryo gender and ploidy. These factors, if ignored, can substantially skew the calculate mtDNA score for a given sample. The outcomes of this study highlighted that mtDNA is an irrelevant marker of embryo viability. More time and further studies, possibly performed in a prospective way, are needed in order to better clarify the usefulness of mtDNA evaluation and the potential definition of a threshold value, in order to identify the most viable embryos.

**Metabolomic profiling**
Over the last decade, the development of non-invasive embryo evaluation focused on the examination of embryonic metabolome in spent culture media. During its growth, an embryo uses nutrients and generates metabolite, changing the composition of its own culture media. These changes reflect the activity of embryo metabolism (40). The hypothesis is that the presence and amount of metabolic products secreted by the embryo in culture media, such as glucose, lactate, pyruvate and amino acid, could provide information on embryo viability (41-45). Metabolomic allows to characterize thousands of metabolites and, depending upon the platform employed, it is possible both their identification and quantification (46). Until now, however, only two algorithms were tested in a prospective way and both of them were unable to demonstrate the efficacy of metabolomic in selecting the most viable embryo (47, 48). In both double-blinded randomized controlled trials, 417 and 752 couples, respectively, were divided at the day of oocyte pick-up in two groups: the control group, in which embryo selection was based only on its morphology and the study group, in which embryo morphological evaluations were combined with metabolomic profiling of spent culture media. The ongoing pregnancy and live birth rates obtained were comparable in both groups in both studies, demonstrating that this technology does not improve embryo selection (47, 48). More recently, a prospective cohort study analyzed the spent culture media on day-3 and on day-5 from 148 single transferred blastocysts. Despite of a high degree of individual metabolomic variation, no correlation was found with pregnancy outcome (49). On the contrary, a study performed on 621 frozen-thawed embryos transferred on day 4 revealed that metabolomic profiling of spent culture media is independent of morphology and correlates with implantation potential (50). Discrepancies revealed from all these studies could be ascribable to several confounding factors related to patient demographics, cycle characteristics, stimulation protocols, embryo culture, instrumentation, laboratory procedures, brand of culture media and so on. Taking into account all these factors during the development of a predictive model of embryo viability is a very hard challenge (51). In conclusion, it is clear that metabolomic profiling is still not ready to be clinically applied to human reproduction, due to the high costs, the special expertise required and the lack of a universally applicable algorithm. Anyway, advances in spectroscopic techniques might lead this method to be a useful adjunct to other embryo selection strategies in the future (23).

**Proteomics**
Another possibility to perform a non-invasive embryo selection is to analyze the secretome that is composed by all proteins secreted in spent culture media by the embryo during its development. It has been postulated that the secretome profiling of an embryo could change according to its viability (23). Anyway, despite the advances in proteomic technologies, knowledge on human embryo secretome is still low (45). In addition, several technical aspects reduce the applicability of this method to the field of human reproduction. For example, the limited template’s availability, the low protein concentration in culture media, the deficiency of sensitivity of utilized platforms, the small protein database as well as the presence in almost all culture media of human albumin as protein source (23, 45). Initially, single proteins or molecules were analyzed in order to test their potential as biomarkers, such as human leukocyte antigen G (HLA-G), platelet-activating factor, leptin, agrocranin and ubiquitin. Among these, HLA-G was found to be produced by human oocytes and embryos and has been investigated in a clinical setting due to its association with successful pregnancy outcome (52). Anyway, several multicentre trials did not confirm these findings (53-55) demonstrating that HLA-G does not improve embryo selection compared to standard morphology evaluation. Due to the multifactorial nature of embryo development, the protein profiling focused on other method such as mass
spectrometry (MS). Katz-Jaffe and collaborators (56) were the first to successfully analyze the proteins secreted in spent culture media from human embryos, correlating them with blastocyst development. They found that through the developmental stages, mammalian embryos change their secrete protein profiles. In the first 24 hours of culture, only maternal proteins were secreted; while on day-3 of development, unique embryonic proteins were detected. In addition, comparison of secretome between developing blastocysts and degenerated embryos showed a significant difference in a 8.5-kDa protein. Its isolation and identification revealed that the best candidate for this biomarker could be ubiquitin that is involved in several processes, such as proliferation, apoptosis and implantation. In 2011 Cortezzi et al. (57) identified 25 proteins in the secretome: 15 and 10 of them were associated with positive and negative pregnancy outcomes, respectively. However, none of these biomarkers were tested in randomized control trials. Apolipoprotein A1 (ApoA1) and hormone human chorionic gonadotrophin (HCG) were also tested by mean of MS technology (58, 59). Increased level of ApoA1 was found in spent culture media from good quality blastocysts compared to poorer ones, although none association with pregnancy outcomes was highlighted (58). Out of four HCG isoforms analyzed, HCGβ was hypothesized to be a potential biomarker due to the fact that it is already present after only two days of culture while HCGh, being found in spent culture media from abnormally fertilized embryos, could be an indicator of pathology. However this study did not analyze any correlation between HCG and pregnancy outcome (59). More recently, Dominguez et al. (60) tried to combine proteomic fingerprinting with time-lapse morphokinetic analysis in order to develop a diagnostic tool able to identify the most viable embryo (60). The combination of seven proteins in the embryo spent media and the timing of embryo development was analyzed. A hierarchic model establishing four categories of embryo with different implantation potential was developed, based on the presence of interleukin 6 and the duration of the second cell cycle in a range of 5-12 hours. In another study, a different model was built after 282 proteins of embryonic origin in the blastocoe of human embryos have been identified and analyzed. In this model the presence of histone H2A and the abundance of the protein GAPDH combined together was able to predict the embryo ploidy (61). Anyway, clinical validation of both these models is mandatory to confirm their efficacy before the introduction in the clinical routine.

In conclusion, even with all its limitations, embryo proteomic analysis remains a promising platform for embryo selection. It is possible to hypothesize that in the near future advancements in the knowledge of the secretome and in the platform’s sensitivity will allow the analysis of single secreted protein at very low concentrations, leading to its clinical application also in IVF centers.

Oxygen consumption

The idea that oxygen consumption (OC) could represent a method enabling the identification of viable human embryo is based on the fact that mitochondria are the main oxygen consumers in human oocytes and embryos and consequently changes in their activity could reflect differences in metabolic activity. In human, OC was demonstrated to be a quality marker of oocyte competence being affected by maternal age and basal FSH concentration (62) or ovarian stimulation protocols (63). Differences in OC rates between fresh and vitrified-warmed blastocysts were also reported, with higher OC in the formers (64). In addition, in vitrified-warmed blastocysts it has been found that OC was restored earlier in hatching or hatched compared to arrested or degenerated blastocysts, suggesting that it could be possible to identify vitrified-warmed blastocysts with high developmental potential on the basis of their respiratory activity (64). In another retrospective study (65), 47,741 OC measurements were performed on 575 injected oocytes obtained in 56 egg donation cycles whit embryo transfer on day-3, with the aim to evaluate the correlation of OC with embryo development and clinical outcomes. Higher OC values were observed in embryos obtained from patients who achieved pregnancy compared to those that did not, independently from the number of implanted embryos; OC values were higher also in implanted compared to not implanted embryos as well as in viable (frozen or transferred) compared to non viable (discarded) embryos. In this study an automated not invasive instrument able to measure OC in controlled conditions (37°C and 5.0% CO₂) of cul-
tured embryos, was employed. Anyway, Authors themselves declared the inability to continue these analysis until future easier devices will be available in order to perform prospective randomized trials. Very recently, a new device it has been set-up, called chip-sensing embryo respiration monitoring system (CERMs), enabling the embryo OC rate to be measured automatically by mean of an electrochemical method (66). In this study, the applicability of CERMs was tested on frozen-thawed human embryos: OC rates of embryos developed until the blastocyst stage were higher compared to arrested embryos after warming. This device shows several advantages: it is an easy, quick and sensitive method, allowing the automatic measurement of OC rate of a single embryo in a minute. In addition, CERMs is a scalable technology that can be integrated into standard or time-lapse incubators, working with reduced gas concentrations. However, despite all these promising features, there are no data on pregnancy outcomes because the transfers of the embryos tested in this study were not performed. Finally, due to limited sample size, further data are needed to validate these findings.

Conclusions

The field of non-invasive screening of embryo quality is a very appealing area of interest in human reproduction. The main challenge in the identification of valid biomarkers for embryo viability is the set-up of a clinically and universally applicable efficient methodology, able to improve clinical outcomes. Some of the most important difficulties are the low platform’s sensitivity, the differences among the centers in laboratory procedures and the lack of knowledge concerning the biological processes of developing embryos. All strategies presented in this review are very promising and it is possible to hypothesize that one or more of them, alone or in combination with each other, could be improved and could be included in a multidimensional approach to embryo selection strategy, leading to enhanced results in the next future. Anyway other well-designed, prospective, randomized and possible multicentric control trials are needed, before their introduction in a clinical routine.

References


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