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Review Article

Pursuit of Markers to Assess and Select Competence of *invitro*-Produced Embryos

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Abstract

Morphological classification has been used as a conventional and non-invasive method to select pre-implantation embryos for transferring to recipients. However, low implantation rate and embryonic mortality after embryo transfer, specially using *invitro* produced or manipulated embryos, indicate than this method is not reliable enough to reflect the developmental competence of an embryo and it needs to be improved for biological and applied needs. In fact, embryo viability and competence correlate better with gene expression pattern than with embryo morphology. Nevertheless, the analysis of gene expression in pre-implantation embryos is an invasive procedure that most frequently implies the lysis of the embryo or a part of it. In this context, the identification of secreted markers linked to embryo quality and development competence may be a useful tool to classify pre-implantation embryos. Candidates may be studied in order to define suitable markers for embryo selection, widely expressed across species and correlated to developmental capacity and survival up to term. This paper presents a review of the literature on the different methods that may be used for embryo scoring as well of those used to predict embryo competence and quality. The most practical methods are those that consider embryo morphology. However, it seems that the molecular signature of each individual embryo is more predictive of its competence and capability to produce a healthy offspring. Many studies and experiments are required to propose a consistent method for embryo selection applicable in the assisted reproductive technologies in both humans and animals.

Keywords: Embryo competence; Embryo selection; Embryo development; ART

Introduction

Establishment and maintenance of a pregnancy able to produce a healthy offspring is the main goal of any reproductive system. In a natural conception, pregnancy lost before the 20th week of gestation reaches 75% in humans and it is attributed to implantation failure [1]. In farm animals, fertility defined as pregnancy rate per cycle might vary from 50 to 80 % and most of pregnancy loss occurs during the first three weeks due to defective embryo development [2-4]. It has been determined that pregnancy success depends on both embryo quality and uterine environment. In fact, embryonic loss is most likely attributed to maternal conditions such as animal age, nutritional factors, stress and uterine infections [3-9] in naturally occurring conceptions. However, *invitro*-produced embryos have a reduced developmental potential, resulting in a low implantation rate and an increase in the frequency of early pregnancy loss. Most of the in-vitro produced embryos are not able to function within a normal development schedule so that embryos can stop developing at any stage. Moreover, some of the embryos that reach the implantation stage do not induce a proper signal for pregnancy recognition and embryo-maternal crosstalk.

The development of Assisted Reproductive Technologies (ARTs) represents a great advance in both commercial and basic studies in animals, as well as in the treatment of infertility in humans [10-16]. However, despite continuous efforts to improve embryo development and competence, competent embryos produced *invitro* nowadays do not exceed 50 % of the total number of fertilized oocytes [17,18], while less than 40 % of transferred embryos produce a healthy offspring [18-21]. In humans, 8 out of 10 transferred embryos will not result in a pregnancy [22]. The low developmental potential of *in-vitro* produced embryos is mainly due to suboptimal conditions provided during oocyte collection and maturation, fertilization and embryo culture [23-25].

Scoring and selection of *invitro*-produced embryos

Morphology as a primary criterion for embryo selection: After *invitro* embryo production, selecting healthy embryos with the best potential to implant and produce an offspring is one of the major tasks for an embryologist. In humans, low embryonic competence is often handled by transferring several embryos to ensure a birth [26]. This practice is frequently the cause of preterm deliveries and other health complications for both the baby and the mother [27]. In farm animals, the main goal of ARTs is to multiply high value animals, which in the long-term contributes to the development of animal agriculture [17]. However, transfers of low competent embryos have a negative economic impact due to the direct costs associated with the maintenance of empty receptors, the price of supplies for embryo transfer and costs of the embryo or fetus lost.

In order to avoid the above-mentioned problems, it is mandatory to perform an accurate embryo selection before transferring. In

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general, morphological parameters are widely used criteria for embryo selection in all species. Morphology and timing of embryo development (the time of first cell cleavage and when embryos reach the morula or the blastocyst stages) are simple and non-invasive for the embryo. In those species in which embryos are transferred at early stages, the morphological selection is based on the Pronucleus (PN) size and location within either the zygote or the number and size of the blastomeres, as well as the fragmentation percentage in later stages (2-8 cells) [28-30]. In humans, some studies have demonstrated that embryo scoring using the PN characteristics might improve embryo selection. However, the use of this parameter is restricted to those species with visible PN at the zygote stage. Embryos from ruminants or pigs have a dark cytoplasm so that visualizing the PN is almost impossible.

When embryos at more advanced stages are transferred, for instance, at the blastocyst stage, criteria such as blastocyst expansion, quality of the Inner Cell Mass (ICM) and Trophoectoderm (TE) and grade of fragmentation are used for embryo classification [31,32]. Gardner and Schoolcraft [31] determined that the pregnancy rate in humans can be grater than 60 % by transferring a blastocyst with an ICM containing many tightly packed cells and a TE with many cells forming a cohesive layer. However, in some cases, blastocyst scoring might be challenging and depend on the subjective criterion of the embryologist. Moreover, the development schedule of *invitro*-produced embryos is very heterogeneous in concordance with their competence. In fact, even grade I embryos are often unable to maintain a normal pregnancy [18,21,33-36]. This statement has been demonstrated in several species; transferring grade I embryos produced by *invitro* fertilization or somatic cell nucleus transfer generate low rate of implantation and development to term [18,21,33-37]. As a concrete example, we found that transferring bovine cloned blastocyst with a very similar morphology (Figure 1) produced only 33 % of pregnancy (day 35) and 11 % of calving [18]. Furthermore, selection of human embryos based on morphology cannot predict chromosome aneuploidies [38]. It is true that blastocyst morphology correlates with the incidence of aneuploidy. However, a high proportion of good and fair human blastocyst are aneuploidy (32 and 41 %, respectively) [37].

From the literature reviewed, it can be inferred that the cumulative data of the morphological parameters of the embryo at different developmental stages might improve embryo scoring and the success of pregnancy [39]. However, embryo evaluation by morphological tools is usually performed at the stage when embryos are transferred since the continuous disruption of embryo culture for evaluation may be detrimental for embryo development and affect its quality and competence negatively. The recorded data from individual embryos during the complete period of embryo culture using a time-lapse system seems to be useful for the selection of human embryos [40,41]. However, this does not constitute a practical tool for *invitro* embryo production in farm animals with commercial purposes due to the high cost of the system, the reduced number of embryos that are checked at a time and the lack of validated programs to follow embryo development parameters of different species.

Despite the number of studies and experiments to improve the use of morphology as a reliable method for embryo scoring, to date, pregnancy outcome is still low in order to satisfy the reproductive needs especially in animals of economic value. In that sense, new strategies have been developed to be used in combination with morphological criteria.

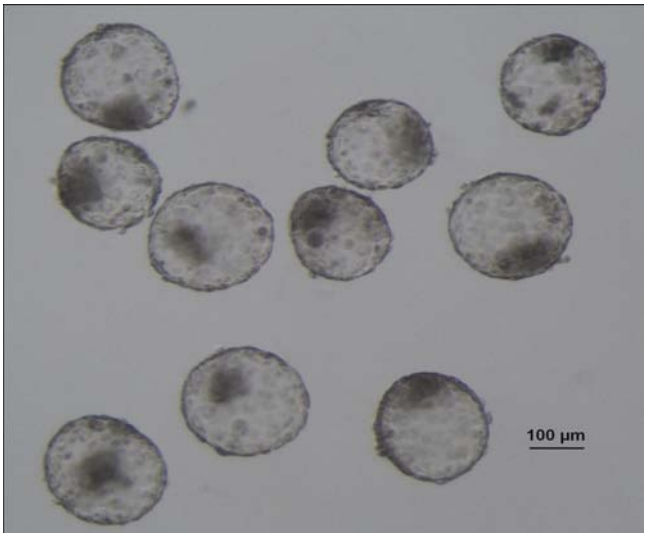

Figure 1: Cloned blastocyst morphologically classified as Grade I, selected to be individually transferred embryo to synchronized recipients. Embryos were produced by Hand Made Cloning, using a cell line from a Red Angus cow as a nucleus donor. The rate of embryo development to blastocyst stage was 62.2 % and 72.8%of the total blastocysts were classified as Grade I. The mean blastocyst diameter was 216.4 µm [18].

Emerging strategies for embryo analysis and classification

The competence differences observed in embryos produced *invitro* can be associated with their molecular signature, including epigenetic modifications what will result in different gene expression patterns, metabolism and response to other manipulation, like cryopreservation [42]. Besides the morphology, the expression patterns of selected genes, the embryonic metabolism and secretome are emerging as interesting methods for embryos scoring and selection.

Improvements in technologies for embryo manipulation have facilitated the development of methods for embryo biopsy, which combined with accurate molecular techniques, allow for the use of DNA or RNA from few cells for genetic diagnosis and gene expression analysis. Currently, blastocysts might be biopsied for genomic DNA or gene expression analysis to test their further developmental potential in livestock species and humans [43-49]. However, the challenge of this technique is the identification of genetic markers that correlate with the developmental capability of the embryo and its potential to produce a viable offspring.

During the few last years, most of the efforts to establish a method for a consistent embryo selection have been based on the study and identification of gene markers, predictors of embryo quality, competence and implantation capability [18,45,50]. In addition, research has been focused on identifying genes that are commonly affected by the *invitro* conditions and that might be responsible for the low competence of *invitro*-produced embryos. As a result, there is an extensive list of papers that mention genes that are aberrantly

expressed during the pre-implantation period as a consequence of the embryo production method, the embryo culture system, among others. However, no convincing markers had been firmly set yet [18,21,35,42,49,50].

For example, it have been demonstrated that canonical pluripotency markers (OCT4 (octamer-binding transcription factor 4), SOX2 (Sex determining Region Y-box 2and NANOG (Homeobox protein NANOG), responsible for the maintenance of the pluripotency of the ICM in bovineblastocyst, might be good candidates to predict embryo competence [18,50]. Bovine blastocysts produced *invitro* showed a marked deregulation of the expression levels of OCT4 and SOX2, while the expression level of these markers had a high variability between individual embryos regardless of the morphology (all embryos were morphologically classified as grade I) [18]. Moreover, when those embryos are transferred to a cattle recipient, not all of them are able to elongate. Nevertheless, when transferred embryos were recovered at Day-17 of development, all elongated embryos displayed correct expression of both markers [18]. This suggests that only blastocysts expressing normal levels of OCT4 and SOX2 are more likely to reach a further development, at least up to the elongation stage. In another experiment, it has been demonstrated that bovine cloned blastocysts with higher levels of OCT4 expression have a better morphological quality at the blastocyst stage and have a higher implantation rate [18] compared with blastocysts that expressed lower levels of OCT4. These results might indicate that the combination of genetic markers with the morphology parameters could be a good approach to predict embryo competence. However, further research is required to confirm these results since the higher implantation rate obtained is still very low [18]. El-Sayed and coworkers [45] performed a large-transcriptome analysis from blastocyst biopsies to determine candidate genes related to embryo developmental competence judged by the outcome of pregnancy. In this study, authors found a long list of differentially expressed genes between low competent blastocyst (resulting in no pregnancy, resorption and abortion) and competent blastocyst (resulting in calf delivery). Nevertheless, it is difficult to select a suitable marker as a predictor of embryo competence within a long list of candidates and several disrupted signaling pathways.

Apart from the lack of proper gene markers, the analysis of gene expression in pre-implantation embryos is an invasive procedure that implies the manipulation of the embryo for the lysis or a part of it. The invasive nature of this approach may be detrimental for the embryo and does not offer a reliable test for embryo quality.

Nowadays, non-invasive strategies are emerging especially in humans [51-53]. Those methods include the transcriptomic analysis of the cumulus cells at the moment of oocyte retrieval; from that, some gene markers related to embryo competence and successful pregnancy were identified [52]. This method seems to be useful in humans; only few oocytes are collected per patient but in animals, where hundreds of oocytes are processed at a time, may become unpractical. In this sense, the analysis of the embryo secretome (metabolites and proteins secreted to the culture medium) may provide a non-invasive method for assessing embryo quality with practical application. The study of embryo metabolism has revealed exaggerated differences between embryos that result in a pregnancy and those that do not [53,54]. In this context, different molecules have been measured in the culture media of the early embryo, including pyruvate, lactate, glucose, amino acid, oxygen and the proteomic profiling [54-60]. There are many examples in the literature in both humans and animals, supporting the possible use of metabolic profile to select competent embryos by their ability to alter the culture environment. For instance, Gardner and Leese [61] showed that the higher rate of glucose consumption predict the implantation potential of mouse blastocysts. Similar results have been obtained in human embryos, in which the greater glucose utilization positively correlates with the implantation potential of the blastocyst [62]. Lane and Gardner [63] showed that the selection of mouse blastocysts based on their glycolytic rate (around 50%) increases the implantation rate up to 96%.This contrasts with the value obtained when embryos were selected only by the morphology and that reached only20% of implantation.

Interesting information have emerged from these studies, but there some contradictions within the literature specially because different compositions of culture media may provoke stress in the embryo due to an excess of energy substrates or a deficit of essential nutrients [64]. Moreover, the metabolomics experiments require a multidisciplinary team and a proper methodology of analysis and further investigations are required to validate the algorithms used to determine the metabolites in different types and volume culture media [53,62].

The study of proteins produced by the embryo and secreted to the environment is emerging as a powerful tool for the assessment of embryo quality and competence. These studies may contribute to the knowledge of cellular processes of the embryo, including early interaction with the maternal environment. Changes in the transcription profile do not always predict a change in the phenotype (biological process and cellular functions) since not all mRNA will be translated. These facts, along with the advance in proteomic technologies, may expand the non-invasive methods to evaluate embryo competence.

At present, some proteins related to embryo development and competence has been identified. For instance, several reports suggest that leptin might be used as a marker for embryo development. It seems that leptin is involved in the initiation of the establishment of a molecular dialogue between the embryo and the maternal side at the time of implantation in humans and mice [65]. In mice, sheep and pigs, the addition of leptin to the culture medium has a positive effect on pre-implantation embryo development depending on the protein concentration and the embryo stage during the treatment [66-68]. However, previous studies showed that the addition of leptin to the culture media decreases the development of 2-cell mouse embryos in the blastocyst stage [69]. Therefore, inconsistency in the results that describe the beneficial effect of leptin on embryo development might be due to different conditions of the embryo culture and the general protocols for embryo scoring. However, based on the similarities of the leptin system in pre-implantation embryos from mice and humans, it is possible to suggest that leptin presence is required for implantation [66,70-72]. Moreover, it has been shown that competent human blastocysts secrete more leptin compared with developmental arrested embryos [65]. However, there are no functional evidences of the value of leptin to predict embryo implantation and delivery in

any species. Therefore, further evidences are needed to propose this protein as a marker for embryo selection.

Apart from leptin, several reports have indicated an association between the presence of soluble HLA-G (human leukocyte antigen G; a non-classical MHC class I molecule that plays a role in immune tolerance in pregnancy) in the spent culture media with the potential of the embryos to produce successful pregnancies. This result suggests a possible use of this marker to predict quality and implantation success of human embryos [73,74]. However, there are contradictory studies since clinical pregnancies have been obtained by transferring sHLA-G–negative embryos [75].

Mains et al. [76] have recently published an interesting work where they performed a serial study of the spent media of *invitro*-produced human embryos with different morphology. In a first analysis, the comparison of day-4 spent media from good blastocyst and cleavage-arrested embryos, revealed several proteins that differed by at least 1.5 fold (increased or decreased) between both groups. In this experiment APOA1 (apolipoprotein A-I) was within the proteins that increased in good blastocyst. Furthermore, in a second analysis comparing day-5 spent media from the same group of embryos, only APOA1 was identified as higher (1.3 fold) in good quality blastocyst.

Based on the data presented above, it is possible to conclude that very few protein markers are described for embryo selection using a non-invasive method neither in human nor in animals. One of the limitations of this kind of studies is the complexity of the samples, the low amount of proteins in the spent media and the high cost of the experiments and analysis.

This review points to the fact that many studies and experiments are still required to propose a consistent method to select competent embryos produced *invitro*. On the other hand, combinations of different approaches may be an appropriate manner to perform embryo evaluation. Improvement of embryo selection is mandatory to increase the efficiency of the ARTs and the success of implantation and development of *invitro*-produced embryos.

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