

Gene expression analysis and in vitro production procedures for bovine preimplantation embryos: Past highlights, present concepts and future prospects

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Contents

Over the past decades in vitro production (IVP) of bovine embryos has been significantly improved and in particular bovine IVP is now widely applied under field conditions. This in vitro technique provides new opportunities for cattle producers, particularly in the dairy industry, to overcome infertility and to increase dissemination of animals with high genetic merit. Improvements in OPU/IVP resulted in large-scale international commercialization. More than half a million IVP embryos are generated on the yearly basis demonstrating the enormous potential of this technology. These advances and the fact that bovine and human early development is remarkably similar have also prompted the use of bovine embryos as a model system to study early mammalian embryogenesis including humans. Despite all the improvements, embryos generated in vitro still differ from their in vivo derived counterparts. Embryos must adjust to multiple microenvironments at preimplantation stages. Consequently, maintaining or mimicking the in vivo situation in vitro will aid to improve the quality and developmental competence of the resulting embryo. The successful clinical application of the techniques in reproductive biotechnology requires both species-specific clinical skills and extensive laboratory experience. The recent advances in transcriptome profiling have also provided deeper insight into RNA expression and regulation at an unprecedented resolution. The development of these high-throughput DNA sequencing methods has resulted in new approaches for both mapping and quantifying transcriptomes. The aim of this review is therefore to summarize the available data related to gene expression analyses as well as in vitro embryo production procedures and to provide ideas about future concepts.

KEYWORDS

bovine, embryo, gene expression, in vitro production (IVP), oocyte, transcriptome

1 | INTRODUCTION

The birth of the first IVF calf derived from an in vivo matured oocyte in 1981 (Brackets et al., 1982) and the discovery of heparin as capacitating agent for bull sperm in 1986 (Parrish et al., 1986) were two key events, ultimately resulting in efficient IVP systems for not only bovine preimplantation embryos, including in vitro maturation

(IVM) of the immature oocyte to the matured metaphase II stage, in vitro fertilization (IVF), and subsequent in vitro culture (IVC) of embryos to the desired stage of development, preferably the blastocyst stage. The first calves produced entirely from IVM-IVF-IVC were born in 1987 (Fukuda, Ichikawa, Naito, & Toyoda, 1990).

According to the International Embryo Technology Society (IETS) statistics, the number of embryos produced in vitro and transferred

into recipients has increased more than 10 times in the last century and is now approaching the numbers of embryos produced *in vivo* by superovulation (Perry 2017). This indicates that OPU and IVP are considered a reliable and cost-effective technique and have acquired a significant role in cattle breeding.

2 | IN VITRO PRODUCTION

Production of embryos *in vitro* is a three-step process involving IVM, IVF, and subsequent culture of the presumptive zygote to the blastocyst stage (IVC). The procedure of IVP is best-developed in cattle. During maturation, the oocyte must undergo a complex array of cytoplasmic rearrangements that allow her to support subsequent fertilization and initiate embryonic development (Sirard, 2016). Proper maturation of the bovine oocyte from the GV stage to metaphase II takes place within a period of 20–24 hours and is a prerequisite for fertilization and pre-implantation development. Substantial efforts have been devoted to the establishment of non-invasive and non-perturbing means for selecting the most competent oocytes (Fair, 2010; Krisher, 2013; Wrenzycki & Stinshoff, 2013), also at the RNA level. IVF is a complex step whose success requires appropriate oocyte maturation, sperm selection, sperm capacitation and IVF media. *In vivo*, fertilization rates are around 90% for heifers and cows (Diskin & Sreenan, 1980). Treatment of bull sperm prior to IVF generally involves the selection of cells with the highest progressive motility. Furthermore, supernumerary sperm, seminal plasma, cryoprotectants and other factors are removed. One of the most common methods for preparing spermatozoa for IVF is to centrifuge them through a concentration gradient, such as a 45% Percoll mixture layered on a 90% solution. In addition, single-layer centrifugation (SLC) through a species-specific colloid has also been shown to be effective in selecting spermatozoa with good motility, normal morphology and intact chromatin (Gloria et al., 2016; Morrell et al., 2014). An alternative method is the swim-up procedure. The changes a sperm cell has to go through before it can successfully fertilize an oocyte are summarized under the term capacitation (Brackets & Oliphant, 1975). Capacitation is recognized as a complex series of biochemical and physiological reactions (Breitbart, Shalev, Marcus, & Shemesh, 1995), including the expression of hyperactivated patterns of motility and the acquisition of the capacity to respond to signals originating from the oocyte. Successful fertilization is characterized by the extrusion of the second polar body and the formation of the female and male pronucleus. In cattle, the pronuclei are not visible due to the amount of lipid vesicles which is in contrast to the picture in for example humans and mice (Parrish, 2014). IVC of bovine embryos is the last step in IVP and involves approximately 6 days of culture from the presumptive zygote onwards. During the early post-fertilization period, several major developmental events occur in the embryo including (a) the first cleavage division (b) the activation of the embryonic genome, (c) the compaction of the morula, and (d) the formation of the blastocyst.

Previously embryo requirements were not known and a temporary *in vivo* culture in surrogate oviducts of sheep was performed (Lazzari et al., 2010). Oviducts of several other mammalian species including rabbits and mice have also been used to culture bovine embryos *in vivo*. The isolated mouse oviduct has also been employed as a system to culture bovine embryos successfully (Rizos, Ramirez, Pintado, Lonergan, & Gutierrez-Adan, 2010). The success of *in vitro* embryo production techniques demonstrates that it is possible to bypass the oviduct during early development and, to a certain extent, replicate the conditions *in vitro*. However, overall they do not adequately mimic well enough the complex series of development-specific steps for which the oviduct has evolved a unique and dynamic microenvironment (Besenfelder, Havlicek, & Brem, 2012).

The most common media for culturing bovine embryos are variations of the original synthetic oviduct fluid (SOF) medium (Tervit, Whittingham, & Rowson, 1972). IVC conditions have been improved in the last years, mainly by adjustment of media formulations. However, while more than 30% blastocyst formation could be achieved in most culture systems, it soon became obvious that quantity did not always match quality (Lonergan, Fair, Corcoran, & Evans, 2006; Niemann & Wrenzycki, 2000; Wrenzycki et al., 2005) and that serum supplementation was detrimental to embryo/fetal development as one main causal factor of the so-called large offspring syndrome (LOS), characterized by altered embryonic and fetal growth, deviant embryonic and fetal gene expression patterns, and high perinatal losses (Lazzari et al., 2002). A large field study demonstrated that the incidence of LOS was greatly reduced by *in vitro* culture in cell-free and serum-free SOF media (Van Wagendonck-de Leeuw et al., 2000). These observations highlight the importance of the post-fertilization culture environment for the quality of the resulting blastocysts. However, the existence of diverse embryo culture media and methods makes it very challenging to define the optimal components of embryo culture media.

Taken together, bovine IVP is at an advanced stage of development. However, an aspect that may change in the future is automation and miniaturization of the IVP process by better mimicking *in vivo* environment, e.g., using microfluidics (Wheeler & Rubessa, 2017) or an encapsulation technology (Blockeel et al., 2009) to obtain IVP embryos of similar quality as the *in vivo* derived counterparts. Such systems would facilitate the gradual change of the culture medium to meet the precise requirements of the developing embryo and overcome substantial limitations of conventional culture systems. Recently, a culture system for the formation of an *in vivo*-like oviduct tissue substitute from primary oviduct epithelial cells has been developed. This air-liquid interphase (ALI) culture is fully functional in terms of morphological differentiation (polarization, columnar shape, ciliary activity), generates oviduct fluid surrogates and enables embryonic development up to the blastocyst stage without addition of embryo culture medium (Chen et al., 2017). However, the ALI-BOEC co-culture system was much less efficient in supporting blastocyst formation than optimized conventional IVP procedures and the gene expression pattern of the embryos developing on ALI-BOEC was similar to that of conventional IVP embryos (Van der Weijden, Chen, Bauersachs, Ulbrich, & Schoen, 2017). Furthermore,

a 3D oviduct-on-a-chip model has been described. This allowed proper sperm and oocyte interactions, fertilization and completely abolished polyspermic fertilization and parthenogenic activation of oocytes in the absence of added sperm activating factors (Ferraz et al., 2017). However, the success of an IVP laboratory may stem not only from improvements of the IVC per se, but from the entire IVP system (Baltz, 2012; Gardner, 2008; Leese, 2012).

Nevertheless, commercial production of bovine embryos via IVP is now successful in many laboratories around the world.

3 | QUALITY ASSESSMENT IN PREIMPLANTATION EMBRYOS

The ultimate test of the quality of an embryo is its ability to produce live and healthy offspring after transfer to a recipient. Identification of the embryo with the highest potential to implant, establish and maintain a pregnancy is a primary goal in assisted reproduction techniques. Embryo selection methodologies are either invasive or noninvasive and can be applied at various stages of development from the oocyte to cleavage-stage embryos and up to the blastocyst stage. Morphology and the proportion of embryos developing to the blastocyst stage are important criteria to assess developmental competence. Evaluation of embryo morphology remains the method of choice for selecting viable embryos prior to transfer. It is the most practical and clinically useful approach to assess of embryo viability (Van Soom, Mateusen, Leroy, & De Kruif, 2003). A bovine embryo grading system developed previously (Lindner & Wright, 1983) is, with minor modifications, still widely applied in this field and published in the IETS manual. However, embryo morphology alone may not be accurate enough to serve as the sole criterion for the prediction of embryo developmental potential *in vivo*. Culture by incubation in a time-lapse imaging system does not harm embryos compared with the standard IVP protocol and results in similar overall embryo development (Holm, Booth, & Callesen, 2002). A promising system for selecting healthy *in vitro* fertilized embryos in cattle has been suggested recently (Sugimura et al., 2012). Morphokinetic embryo analysis by monitoring the changes in embryo morphology over time, is by far the most important non-invasive embryo selection tool today and is routinely used in human IVF laboratories (Pribenszky, Nilselid, & Montag, 2017). However, due to its high costs it has not entered the commercial veterinary field. An important non-invasive tool to either indicate possible predictive biomarkers of viability or to explain IVP outcome afterwards might be the analyses of spent culture media (Munoz et al., 2014). Recently, much attention has also been given to extracellular vesicles (EVs), which act as mediators of intercellular communication. EVs called exosomes (50–150 nm) and microvesicles (100–1000 nm) are present in ovarian follicular fluid and oviductal fluids (Lopera-Vasquez et al., 2016). These cell-secreted vesicles contain bioactive components (i.e., proteins, lipids, mRNAs and miRNAs). Apart from the role of EVs during follicle and oocyte development, these vesicles can also stem from the oviduct and endometrium, mediating embryo-maternal communication suggesting their role for preimplantation development (Burns et al., 2014; Da Silveira et al., 2017; Lopera-Vasquez et al., 2016).

4 | GENE EXPRESSION ANALYSES IN PREIMPLANTATION EMBRYOS FOR QUALITY ASSESSMENT

Although non-invasive approaches are improving, invasive ones have been extremely helpful in finding candidate genes to determine embryo quality (Graf et al., 2014; Rizos et al., 2008; Wrenzycki, Herrmann, & Niemann, 2007). Techniques to analyze RNA expression patterns have become a powerful tool to determine the abundance of transcripts related to oocyte and/or embryo quality. Numerous efforts to identify candidate genes for the developmental competence of bovine oocytes and embryos have been made employing different strategies. To determine the molecular basis for oocyte maturation and preimplantation development, individual differentially expressed genes of interest must be identified and analyzed in detail. Qualitative differences between *in vivo* and *in vitro* generated bovine morulae and blastocysts were detected for the first time in 1996 using a RT-PCR method (Wrenzycki, Herrmann, Carnwath, & Niemann, 1996). Quantitative RNA analyses were first possible with the implementation of a RT-PCR assay at a semi-quantitative level (Wrenzycki, Herrmann, Carnwath, & Niemann, 1999) followed by the introduction of the RT-qPCR technology. It monitors the amplification of a targeted (c) DNA molecule during the PCR, i.e., in real-time (Bustin et al., 2009). Reverse transcription (RT) coupled with PCR allows the detection of rare transcripts and has been the method of choice for studying individual genes. It is still in use and often also employed for validation of array-based data.

Various technologies have then been developed to deduce and quantify the entire transcriptome, including hybridization- or sequence-based approaches. Hybridization-based approaches are high-throughput and relatively inexpensive (Wang, Gerstein, & Snyder, 2009). However, these methods have several limitations, which include reliance upon existing knowledge about genome sequence; high background levels owing to cross-hybridization; and a limited dynamic range of detection owing to both background and saturation of signals. Moreover, comparing expression levels across different experiments is often difficult and can require complicated normalization methods (Wang et al., 2009).

In contrast to these microarray methods, sequence-based approaches directly determine the cDNA sequence. Recently, the development of novel high-throughput DNA sequencing methods has provided a new method for both mapping and quantifying transcriptomes. This method termed RNA-seq (RNA sequencing) has clear advantages over existing approaches. RNA-seq is the first sequencing-based method that allows the entire transcriptome to be surveyed in a high-throughput and quantitative manner (Wang et al., 2009). Like other high-throughput sequencing technologies, RNA-seq faces several informatics challenges, including the development of efficient methods to store, retrieve and process large amounts of data (Rostom, Svensson, Teichmann, & Kar, 2017). RNA-seq data employing pools of *in vivo* or *in vitro* generated bovine embryos have been reported in 2010 and 2012 (Driver et al., 2012; Huang & Khatib, 2010). The first application of the RNA-seq technology in single *in vitro* produced bovine embryos has been published in 2013 (Chitwood, Rincon, Kaiser,

Medrano, & Ross, 2013). In vivo derived embryos were analyzed one year later (Jiang et al., 2014). The first comparison of embryonic expression profiles across three mammalian species, human, mouse and bovine, for which RNA-seq data were available, clearly show that the three species share more maternally deposited genes than embryonic genome activated genes. More importantly, there were more similarities in embryonic transcriptomes between bovine and humans than between humans and mice, demonstrating that bovine embryos are better models for human embryonic development (Jiang et al., 2015) which has been introduced already earlier (Wrenzycki et al., 2001). The RNA-seq technology has further been used to determine that despite similar morphology and development to the blastocyst stage, preimplantation embryos derived from high and low fertility bulls displayed significant transcriptomic differences (Kropp et al., 2017) demonstrating that the sperm does not only contribute DNA to the oocyte at fertilization. Furthermore, morphologically similar in vivo and in vitro generated bovine blastocysts show transcriptomic variations (Driver et al., 2012). Information of candidate genes regulated by cytoplasmic polyadenylation during oocyte maturation has been provided (Reyes, Chitwood, & Ross, 2015). More recently, transcript profiling of single embryonic cells for a set of candidate genes has been performed for different stages from zygote to blastocyst (Negron-Perez, Zhang, & Hansen, 2017; Wei et al., 2017), providing new insight into lineage specification events in bovine embryos (Lavagi et al., 2018).

Out of the vast amount of non-coding RNA molecules which have been identified recently (Mattick & Makunin, 2006), microRNAs (miRNAs) are short non-coding RNAs of 19–24 nucleotides. They are interesting candidates for maternal mRNA degradation because they regulate gene expression in a temporal- and spatial-specific manner (Hossain, Salilew-Wondim, Schellander, & Tesfaye, 2012; Mondou, Dufort, Gohin, Fournier, & Sirard, 2012). More recently it has been shown that miRNAs are also present in body fluids (Kropp, Salih, & Khatib, 2014). Studies have shown that miRNAs are selectively secreted from cells; however, they may be secreted in various forms such as exosomes or apoptotic bodies (Rayner & Hennessy, 2013; Xu, Yang, & Ai, 2013). Differential miRNA gene expression was observed between embryos that developed to the blastocyst stage and those that failed to develop from the morula to blastocyst stage. MicroRNAs are also secreted from pre-implantation embryos into culture media and their expression may correlate with the developmental competence of the embryo. Expression of miRNAs in in vitro culture media could furthermore allow for the development of biological markers for selection of better quality embryos and for subsequent successful pregnancy (Kropp & Khatib, 2015).

Finally, as epigenetic changes can be induced by environmental factors, understanding how vitro production conditions can interfere with these processes is of critical importance. The embryonic epigenome will continue to be an important area of research, especially to gain a better comprehension how the epigenome influences short- and long-term health. A better understanding of the mechanisms and the role of epigenetics during early embryogenesis will likely improve in vitro protocols and ultimately animal health and productivity.

Epigenetic remodeling during preimplantation development is complex and dynamic, including changes in DNA methylation and histone modifications that occur both on a global scale but also differentially at specific loci. Uncovering the bases of these mechanisms will improve our understanding of early development and are promising for improving animal fertility and diagnosing and treating infertility problems (Canovas & Ross, 2016; Urrego et al., 2015).

5 | CONCLUSIONS

Despite the large number of publications in the field it is still a long way to better understand and manipulate the mechanisms controlling oocyte maturation and early embryonic development. Overcoming these gaps may allow the improvement of ART results. In particular, studies aiming at finding effective biomarkers for oocyte and embryo competence are urgently needed. The field of OMICs seems to be quite promising, especially regarding new findings in transcriptomics, proteomics and lipidomics in oocytes, cumulus cells, embryos and in the EVs. Future studies on this subject might enable the design of more complex, defined and efficient culture conditions for oocytes and embryos.

CONFLICT OF INTEREST

The author has no conflict of interest to declare.

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