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Strategies to increase in vitro embryo yield: lessons from cell and molecular research

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Abstract

Numerous reproductive biotechnologies are commonly employed to enhance animal production mainly through multiplying animals with high-quality traits in a large-scale production system. There is, however, several peculiarities during the process of embryo in vitro production that are still in need of further studies in order to obtain a higher efficiency. This present review discuss some of such particularities, as well as new models of embryo and gamete production, which will probably be part of a new era of reproductive biotechnologies in a near future.

Keywords: animal reproduction, bovine, embryo, *in vitro* studies.

Introduction

The development of embryo technologies have been for long time challenging technicians to find better strategies and apply them in an efficient and low-cost manner, aiming benefits to animal production. Having such opportunities in mind, it is due to our society to face the challenges of improving such technologies, and also, to be ready and able to develop and adapt new technologies.

Last decades have witnessed a huge development of biotechnology methodologies, elaborated or else adapted for our specific conditions, such as embryo transfer (ET), *in vitro* fertilization (IVF), fixed time embryo transfer (FTET), and also some cryopreservation approaches that allow embryo large-scale production.

Theoretically, an efficient embryo production leads to a multiplying process of specifically selected animals and consequently, to an improvement on animal breeding. However such improvement is still not fully described in scientific reports or studies containing clear and evident examples.

Which would be the reasons? Would we have reached a plentiful competence so that such studies will not have to be performed? Are there still opportunities for the improvement of gamete generation or embryo production derived from selected animals?

Oocyte donors

One of the most prominent properties in the process of embryo production is the individual variation between donors. Such feature is highly noticeable and observed in both embryo or cumulus-oocytes complexes (COCs) recovery by superovulation/embryo transfer or ovum pick-up (OPU) procedures (Yang *et al*., 2008; Pontes *et al*., 2011).

The number of embryos produced by OPU/IVF is associated only to folliculogenesis and independent from uterine-environment or males, allowing therefore a very unbiased research strategy.

Indeed, it has been reported that oocyte recovery from some specific animals may be higher when compared to other animals, with a distribution similar to a Gaussian curve. Moreover, there is a tendency for such higher recovery to be maintained throughout several follicle aspirations sections (Garcia and Salaheddine, 1998). There are also reports showing that the pool of preantral follicles in a bovine ovary presents high correlation with the number of antral follicles found, and most importantly, with its fertility (Mossa *et al*., 2012). Finally, discussions with colleagues that use OPU in a routine manner leads to the hypothesis that such characteristic is genetically transferred and that daughters of females that present a pool of increased antral follicles frequently present the same characteristics.

A study to observe such genetic characteristic was recently developed aiming the identification of molecular markers related to the number of viable COCs retrieved (Santos-Biase *et al*., 2012). Even though few markers were evaluated, they have showed significant effect on COCs production, and 1.9 more COCs could be retrieved per OPU routine. Interestingly, the markers were present more frequently in zebu animals, and therefore may be associated to a higher OPU/IVF efficiency in these species. Therefore, studies applying large-scale embryo *in vitro* production systems are encouraged and may contribute to the increase of embryo production.

Oocytes transcripts and competence

The oocyte competence to go through fecundation and to properly develop into blastocyst and term strongly depends on the synthesis and storage of several components during the oogenesis (Lonergan *et al*., 2003; Gandolfi *et al*., 2005; Sirard *et al*., 2006). It is well established that such components (i.e. RNAs, proteins, and energetic substrates) are essential during

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the earliest stages of embryo development, when their transcriptional activity is limited (Memili *et al*., 1998; Picton *et al*., 1998; Memili and First, 2000; Meirelles *et al*., 2004). In oocytes, gene expression is regulated and variable throughout oogenesis. Differentially expressed genes have been characterized in oocytes with greater developmental competence (Pan *et al*., 2005; Fair *et al*., 2007; Katz-Jaffe *et al*., 2009; Mamo *et al*., 2011). These findings support the hypothesis that specific mRNAs and proteins produced during oogenesis drive the adequate embryo development (Robert *et al*., 2000; Fair *et al*., 2004; Gutiérrez-Adán *et al*., 2004; Meirelles *et al*., 2004; Mourot *et al*., 2006; Patel *et al*., 2007; Caixeta *et al*., 2009; Romar *et al*., 2011). It is of general consensus that oocytes that do not complete the process of mRNAs synthesis until ovulation or OPU have poorer developmental competence (Fair *et al*., 1995). In cattle, oocytes from COCs morphologically classified as grade 1 quality have greater amounts of mRNAs than poorer quality oocytes (Biase *et al*., 2008). However, any correlation between mRNA amount and the developmental capacity to blastocyst was observed when the same oocyte was used for both the analysis of the quantity of transcripts and the developmental rate (Biase *et al*., 2009). The feasibility of such an experiment required the creation of a new retrospective model that allowed the individual analysis of development. For that, the transcripts were quantified from cytoplasmic biopsies of mature oocytes parthenogenetically activated and individually cultivated (Biase *et al*., 2009). Although these findings indicate that the global amount of mRNAs is not related to the embryo competence of development, more than a hundred genes were already described as differentially expressed between competent and incompetent oocytes (Robert *et al*., 2000; Fair *et al*., 2004; Gutiérrez-Adán *et al*., 2004; Mourot *et al*., 2006; Patel *et al*., 2007; Caixeta *et al*., 2009; Romar *et al*., 2011). Thus, this same retrospective model described above was used to analyze differentially expressed genes between oocytes that have developed into blastocysts and those that were blocked at the 8-16 cells stage (Biase *et al*., 2012). Twenty-nine genes were identified as differentially expressed, 16 of those have higher expression in the biopsies of the oocytes that developed into blastocysts, and 13 in the ones that were blocked at the 8-16 cells stage. A significant part of these genes were involved in the regulation of transcription, RNA processing and protein synthesis and degradation (Biase *et al*., 2012). Moreover, other differentially expressed genes related to DNA repair and replication and also in the cellular cycle were detected (Biase *et al*., 2012). Thus, these findings are in agreement with previous reports that indicate that the transcripts stored during the oocytes growth and maturation are important to determine their developmental competence (Robert *et al*., 2000; Fair *et al*., 2004; Gutiérrez-Adán *et al*., 2004; Mourot *et al*., 2006; Patel *et al*., 2007; Caixeta *et al*., 2009; Romar *et al*., 2011; Ripamonte *et al*., 2012).

Developmental competence and number of copies of mitochondrial DNA

During the oogenesis and folliculogenesis the number of copies of mitochondrial DNA (mtDNA) increases significantly, ending up with mature oocytes with hundreds of thousands of copies (Cao *et al*., 2007; Shoubridge and Wai, 2007; Cree *et al*., 2008; Wai *et al*., 2008). The oocyte content of mtDNA is greater than what is found in any other cellular type (May-Panloup *et al*., 2007), driving the attention to a potential importance of mitochondria for fertilization and early embryo development (Smith *et al*., 2005; May-Panloup *et al*., 2007; Shoubridge and Wai, 2007). Thus, oocytes with reduced number of copies of mtDNA may have poorer development or incapacity to develop after fertilization compared to oocytes with normal content of mtDNA. Despite their huge content, the number of copies of mtDNA considerably varies between different oocytes (Tamassia *et al*., 2004; Smith *et al*., 2005; May-Panloup *et al*., 2007; Chiaratti *et al*., 2010a). Although it remains unclear what determines such variation or its effects upon development (May-Panloup *et al*., 2007), several studies have related the amount of mtDNA and fertility in many species (Reynier *et al*., 2001; May-Panloup *et al*., 2005a, 2007; El Shourbagy *et al*., 2006; Santos *et al*., 2006; Wai *et al*., 2010). In humans, reduced amount of mtDNA was found in unfertilized oocytes with intrinsic abnormalities compared to oocytes that failed to fertilize because of impairments related to other factors (Reynier *et al*., 2001). Although it seems evident that the depletion of the content of mtDNA is associated with oocyte disruption of competence (May-Panloup *et al*., 2007), this relation is still controversial (Chiaratti and Meirelles, 2010; Chiaratti *et al*., 2010a).

The same retrospective model described below was used to investigate the occurrence of a correlation between the number of copies of mtDNA and oocyte competence (Chiaratti *et al*., 2010a). However, no differences were observed regarding the number of copies of mtDNA of oocytes that normally developed into blastocysts and the ones that blocked or lacked cleavage. Even the oocytes with 90% less mtDNA than average were capable of reaching the blastocyst phase (Chiaratti *et al*., 2010a). These findings are contradicting with the general consensus that the amount of mtDNA affects oocyte competence to develop into blastocyst (Reynier *et al*., 2001; May-Panloup *et al*., 2005a, 2007; El Shourbagy *et al*., 2006; Santos *et al*., 2006; Wai *et al*., 2010). A subsequent study was then designed to evaluate the developmental capacity of oocytes subjected to mitochondrial depletion (Chiaratti *et al*., 2010a). Oocytes were centrifuged to concentrate the mitochondria in one extremity and allow the removal of the mitochondria-enriched cytoplasmic

fraction by aspiration. When the depleted oocytes were cultured they developed into blastocysts in similar rates of intact-control oocytes (Chiaratti *et al*., 2010a), supporting our previous finding. Molecular analysis performed in the resulting embryos evidenced that the content of mtDNA is restored during its development to blastocyst when the oocyte is mitochondrial depleted (Chiaratti *et al*., 2010a). The mtDNA reestablishment is accompanied by the overexpression of genes enrolled in the control of mtDNA replication (Chiaratti *et al*., 2010a). This result suggests that when the oocytes have reduced number of copies of mtDNA (i.e. oocytes with 90% less mtDNA and capable to develop into blastocyst), the embryo is able to regulate the replication of the mitochondrial genome to attempt the energetic demands of the pre-implantation period (Thompson, 2000; Houghton and Leese, 2004; Dumollard *et al*., 2007; May-Panloup *et al*., 2007). This finding is opposed to previous studies that point out a relationship between mtDNA content and oocyte competence (Reynier *et al*., 2001; May-Panloup *et al*., 2005a, 2007; El Shourbagy *et al*., 2006; Santos *et al*., 2006; Wai *et al*., 2010). The contradiction of results may lay in different causes. First, the level of mtDNA in mice is kept constant during the pre-implantation period (Smith *et al*., 2005; Thundathil *et al*., 2005; Cao *et al*., 2007; Cree *et al*., 2008; Wai *et al*., 2008), differently from what is found to occur in cattle (May-Panloup *et al*., 2005b; Smith *et al*., 2005). This can explain the connection found between the oocyte competence and the content of mtDNA in mice (Wai *et al*., 2010). In humans, it is possible that the reduced number of copies of mtDNA observed in unfertilized ova is consequence of a disruption in the machinery of mtDNA replication (Reynier *et al*., 2001; May-Panloup *et al*., 2005a; Santos *et al*., 2006). On the other hand, in cattle, the oocytes subjected to the depletion of a cytoplasmic fraction concentrated with mitochondria were probably free of abnormalities and consequently able to restore their original stocks of mtDNA by activating the machinery of replication. Thus, the embryonic development was unaffected by initial depletion (Chiaratti *et al*., 2010a). Abnormalities in the machinery of mtDNA replication were already reported in humans (Luoma *et al*., 2004; Pagnamenta *et al*., 2006). One example is the alteration in the expression of *TFAM,* a key gene that regulates mtDNA replication (Smith *et al*., 2005; May-Panloup *et al*., 2007).

Cytoplasmic transfer as a tool to restore oocyte competence

The cytoplasmic transfer was used in the late nineties to improve the results of assisted reproduction in women with recurrent fails in embryonic implantation after ICSI (intracytoplasmic sperm injection) or IVF (Cohen *et al*., 1997, 1998; Huang *et al*., 1999; Lanzendorf *et al*., 1999). By this procedure, 5 to 15% of the cytoplasm from a presumed competent oocyte was transferred during the ICSI to the oocyte of a patient with fertility problems. This technique allowed the reestablishment of oocyte capacity to develop into a viable embryo and culminated in the delivery of healthy babies (Cohen *et al*., 1997, 1998; Huang *et al*., 1999; Lanzendorf *et al*., 1999). Thus, it has been suggested that one or more cytoplasmic factors transferred during this procedure were responsible for rescuing development by sustaining the necessities of the incompetent oocyte (Barritt *et al*., 2001; Chiaratti *et al*., 2011b; Levy *et al*., 2004; Poulton *et al*., 2010). In domestic animals, the cytoplasm transfer has been more frequently used as a model to study mitochondrial inheritance (Steinborn *et al*., 1998; Levy *et al*., 2004; Chiaratti *et al*., 2010b; Ferreira *et al*., 2010; Sansinena *et al*., 2011). However, considering the importance of cytoplasmic inheritance to the early embryonic development (Picton *et al*., 1998; Meirelles *et al*., 2004; May-Panloup *et al*., 2007; Shoubridge and Wai, 2007), it can be potentially used to improve the fertility of animals with oocytes of reduced fertility. This may be applied, for example, to restore oocyte competence of repeat breeders Holstein cows. Recently, the low fertility of repeat breeder cows exposed to heat stress was attributed to oocyte disruption, with indicatives of cytoplasmic alterations (Ferreira *et al*., 2011). If results are confirmed, the competence of development of oocytes recovered from repeat breeder cows can be potentially restored by the supplementation with a fraction of cytoplasm from oocytes of categories known as more fertile (i.e. Holstein heifers).

Based on the fore discussed data, several cytoplasmic factors can be responsible for developmental failures of incompetent oocytes (Picton *et al*., 1998; Robert *et al*., 2000; Meirelles *et al*., 2004; May-Panloup *et al*., 2007; Biase *et al*., 2008, 2009, 2012; Chiaratti and Meirelles, 2010). Among these, the importance of the number of mtDNA copies per oocyte has been most discussed once it probably represented a limiting factor for its competence when cytoplasmic transfer was successfully used in humans (Barritt *et al*., 2001; Levy *et al*., 2004; Chiaratti *et al*., 2011b). It is reasonable to consider that cytoplasm transfer introduces mitochondria in the recipient oocyte, what may restore its developmental competence by enhancing the content of mtDNA when this is limiting (Barritt *et al*., 2001; Levy *et al*., 2004; Chiaratti *et al*., 2011b). However, due to previous discussed data rejecting the relation between mtDNA content and oocyte competence (Chiaratti and Meirelles, 2010; Chiaratti *et al*., 2010a), we believe that this hypothesis is strongly questionable for cattle. Aiming to investigate this hypothesis, bovine oocytes were incubated with etidium bromate (EtBr) during the IVM (Chiaratti *et al*., 2011a). The EtBr is known for its capacity of interfering on mtDNA replication, resulting in depleted copies in somatic cells (Chiaratti and Meirelles, 2006). When

oocytes treated with EtBr were fertilized, a decrease on blastocyst development was observed. This decrease was completely reversed by cytoplasmic transfer from oocytes that were not exposed to EtBr (Chiaratti *et al*., 2011a). Hence, when the embryos that had their development capacity restored by cytoplasmic transfer were transferred to synchronized recipient cows, healthy calves were born (Chiaratti *et al*., 2011a). Unexpectedly, neither the number of copies of mtDNA nor the mitochondrial function estimated by the analysis of the mitochondrial membrane potential nor the total amount of ATP were altered in EtBr-treated oocytes (Chiaratti *et al*., 2011a). Because the treatment with EtBr can have affected another cytoplasmic components (i.e. RNAs, proteins, energetic substrates), the effect of EtBr on development may not be resulted by mtDNA replication disruption (Malter, 2011). Anyway, these results clearly demonstrated that cytoplasmic transfer can successfully restore the competence of compromised oocytes (Malter, 2011). Thus, the potential use of cytoplasmic transfer to improve the competence of oocytes to develop in viable blastocysts was reaffirmed. This technique can be an interesting strategy to restore fertility of females with low embryos yield.

Perspectives on *in vitro* embryo production

Increasing nuclear transfer embryo production and generation of gametes in vitro

Reproductive biotechnologies have for long shown its use for the production, selection, and multiplication of valuable animals.

Cloning through nuclear transfer, indeed, is one of the biotechnologies discussed since the birth of the ewe Dolly, the first mammal derived from a somatic cell nuclear transfer (SCNT; Wilmut *et al*., 1997). After Dolly, several other animal species were already produced in laboratories throughout the world.

SCNT provides the possibility of the oocyte cytoplasm to reprogram an already differentiated cell into a pluripotent status similar to the embryonic one, and then to generate a new organism. The factors present in the ooplasm, as well as their exact mechanism of action during the reprogramming of the differentiated nucleus are not yet fully known.

Particularly on farm animal production, the possibility of generating identical individuals is highly desirable when two main concerns are considered: the first, which has been employed since SCNT generation until now, is the production of high quality herds and animals carrying special characteristics. Such selected animals would be multiplied by large-scale cloning, evaluated, distributed to breeders or owners, and used in specific reproductive systems.

However, the second concern, which is probably the most striking opportunity enabled by cloning regarding animal production, has not yet been accomplished. The large-scale production of identical commercial herds in a defined breeding system, similar to other livestock presenting homogeneous genetic lineages, and its derived products commercialized to specialized food or pharmaceutical companies, is still not viable nowadays due to the low efficiency of SCNT.

Although SCNT reprograms a differentiated nucleus, it remains an inefficient technique. Less than 5% of produced embryos generate healthy adult animals (Wilmut, 2002; Cibelli, 2007). Several studies have demonstrated nuclear reprogramming deficiencies in cloned embryos (Bourc'his *et al*., 2001; Dean *et al*., 2001; Rideout *et al*., 2001; Santos *et al*., 2003) leading to problems such as placental dysfunctions, large offspring syndrome, and hepatic and respiratory complications (Hill *et al*., 1999; Heyman *et al*., 2002; Meirelles *et al*., 2010).

As mentioned before, the factors that determine the ability of the oocyte cytoplasm to reprogram the somatic cell nucleus have been under investigation. It is reported that cloning efficiency is inversely correlated to the differentiated status of the donor cell, suggesting that an undifferentiated nucleus is more likely to be remodeled and reprogrammed (Green *et al*., 2007). Hence, cloning using embryonic stem cells (ESC; Rideout *et al*., 2000; Humpherys *et al*., 2001) have resulted in higher efficiency (Hiiragi and Solter, 2005) when compared to the use of more differentiated cells such as lymphocytes (Inoue *et al*., 2005) and fibroblasts (Wakayama *et al*., 1999; Humpherys *et al*., 2001; Wakayama and Yanagimachi, 2001; Hochedlinger and Jaenisch, 2002; Gong *et al*., 2004; Blelloch *et al*., 2006). Therefore, the selection of cell populations that are amenable to reprogramming, for example, the use of ESC as donor nuclei, may be important to increase the cloning efficiency (Solter, 2000).

Interestingly, embryonic stem cells derived from other species other than mouse and humans, for example farm animals, fail to maintain the pluripotent characteristics *in vitro*. Despite innumerous studies, they still lack consistency of pluripotency markers and have not produced chimeras. These cells, are, therefore named stem cells-like, are not able to maintain its characteristics during *in vitro* culture, hampering its use for cloning.

The use of truly pluripotent cells which can be maintained *in vitro* for long periods without losing pluripotency properties as nuclei donors on animal cloning, however, was enabled by the advent of genetic induction of differentiated cells into pluripotency after forced expression of pluripotency-related transcription factors (*OCT3/4*, *SOX2*, *KLF4,* and *C-MYC*, represented by OSKM; Takahashi and Yamanaka, 2006, Takahashi *et al*., 2007). These cells, called induced pluripotent stem cells (iPS *cells* or iPSC) are actually a groundbreaking advent for stem cell research in farm animals, once controversially to ESC, iPSC can be

generated and *in vitro* cultured in these species, and moreover, they apparently show pluripotency patterns similar to those from human and murine ES cells.

Recent data from our lab indicate that bovine fetal fibroblasts can be *in vitro* reprogrammed into pluripotent after lentiviral transduction of murine OKSM (unpublished data). These cells were positive for several markers of pluripotency, therefore being characterized in a pluripotent status not yet reported in bovine ESC-like (Fig. 1).

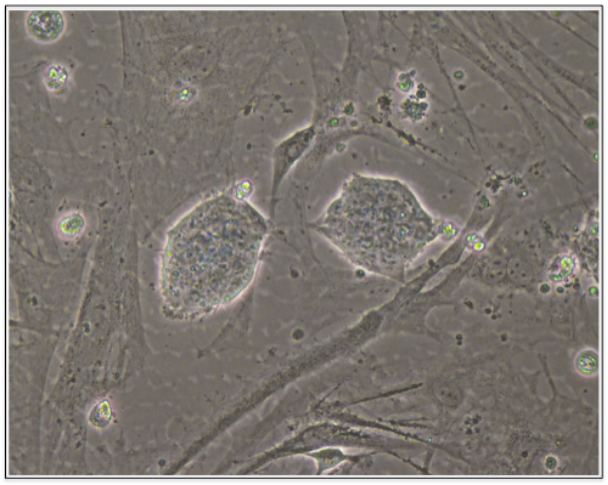

Figure 1. Bovine iPSC. 200X.

Recently the production of cloned swine and mice embryos after nuclear transfer of iPSC as nuclei donors was reported (Cheng *et al*., 2012; Liu *et al*., 2012). In our conditions, when bovine IPS (biPS) cells were used as nuclei donors, they were able to derive pre-implantation embryos. When these embryos were transferred to recipient cows, at least initial pregnancies could be established. However, cell cycle synchronization between biPS and oocyte needs to be optimized in order to allow a real comparison between developmental rates of embryos produced *in vitro*.

The ability of in vitro gamete generation

Another remarkable evidence that *in vitro* embryo production may benefit from nuclear reprogramming processes is the possibility of *in vitro* generation of functional gametes. Epigenetic studies conducted mainly based on cloned and induced reprogramming models have helped the ability of iPSCs in producing functional gametes, which may be helpful not only for the purpose of autologous treatment of several animal or human infertilities, but also, may avoid developmental problems found in SCNT-derived embryos.

When properly cultivated and maintained ES or iPS cells have been shown to be able to generate structures similar to primordial germ cells (PGCs; Hubner *et al*., 2003; West *et al*., 2006). The induced PGCs were able to develop into structures similar to oogonia, which are able to undergo meiosis, to recruit adjacent cells to form follicles and mediate the development to blastocyst after spontaneous parthenogenesis (Hubner *et al*., 2003; Dyce and Li, 2006).

Female or male gamete-like structures have been derived *in vitro* in humans, mice and swine (Nagano, 2007). Recently, the generation of viable animals after induction of ES or iPS cells into PGCs-like cells *in vitro* was reported. These cells were able to develop into gametes *in vivo*, which were recovered, submitted to IVM and IVF in mice (Hayashi *et al*., 2012).

Cellular reprogramming in germinal cells is still a rare event, and similarities between such process and natural reprogramming need further studies. *In vitro* generation of functional gametes derived from other cell types from selected animals, without presenting the complications due to SCNT, may lead to a huge improvement on reproductive biotechnologies related to *in vitro* embryo production in a near future.

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