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New applications for bovine IVP technology: from 'single oocyte culture' to toxicity screening

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Abstract

Bovine *in vitro* embryo production (IVP) has been around for about three decades. Over the years, it has gained a reputation as one of the most applicable of the high-tech bovine assisted reproduction techniques. It is well known that its ups and downs are basically driven by economic imperatives depending on the agricultural system in which it is used. In parallel, there has always been a strong interest in the bovine embryo as an *in vitro* model for basic research purposes. This mini-review focuses on a few more recent applications of bovine IVP that can be useful for both cattle breeding business and research purposes, namely the development of a single oocyte culture system and, as an example, its possible use as a toxicity screening tool.

Keywords: bovine *in vitro* embryo production (IVP), cattle, (single) oocyte culture, toxicity screening.

Introduction

Bovine *in vitro* embryo production (IVP) has been routinely used for almost 25 years and has dramatically changed cattle breeding industry. As substantiated by several data sets, it is clear that the focus of the cattle breeding industry using assisted reproductive techniques shifted towards large world players in Latin America, Australia and the Far East, although it is difficult to get accurate data from the Asian market (Stroud, 2011; Merton, 2011). Based on the available statistics of production, the importance of bovine IVP has declined proportionally in Europe. Parallel to the shift on the economic importance, the research on applied assisted reproduction techniques has also boosted in cattle producing countries, which is clearly illustrated by the increasing amount of scientific publications on IVP in international journals over the past decades (Goovaerts, 2012).

These economical changes impose the creation of alternative applications for bovine IVP. In this context, the possible role of the bovine *in vitro* produced embryo (and cattle reproduction in general) as a model for the study of human (assisted) reproduction strategies has gained an enormous interest from the international scientific community (Campbell *et al*., 2003; Malhi *et al.*, 2005; Adams *et al*., 2012). Bovine and human embryos are remarkably similar with respect to microtubule formation during fertilization, the timing of genome activation, metabolic requirements, interactions with the culture medium and duration of pre-implantation development (Navara *et al*., 1995; Anderiesz *et al.*, 2000; Ménézo *et al.*, 2000). This short review highlights a few new applications of bovine IVP, namely the development of a routine 'single oocyte' culture system and one of its possible uses, the bovine embryo as a tool for toxicity screening.

The need for a bovine 'single oocyte' culture system

Bovine IVP has permanently changed cattle breeding business, as excellently reviewed by others (Hasler *et al*. 1998; Galli *et al*. 2001; Merton *et al*., 2003). Generally, in routine bovine IVP, embryos are cultured in groups. Once the retrieved oocytes are grouped for maturation, the link between the individual oocyte and its specific follicular microenvironment and the donor's physiological history is lost. Although this link can be extremely important for further oocyte development (Vassena *et al*., 2003), the only non-invasive quality parameter left at this stage is COC morphology. Tracking individual oocytes and studying factors that influence their quality and developmental capacity requires two important procedures: individual or 'single' oocyte culture conditions that permit to follow oocytes individually and 'over time', non-invasive quality assessment techniques of immature oocytes. Unfortunately, group culture is still a prerequisite to achieve acceptable blastocyst rates in all intensive IVP systems, since it has been demonstrated that the presence of quality grade I oocytes can facilitate the development of COCs with a lower developmental potential (Khurana and Niemann, 2000). While testing (new) oocyte quality assessment methods in a group culture, the positive or negative effects from neighboring developing and/or degenerating oocytes can bias conclusions on the developmental competence of individual oocytes.

Apart from being a tool to test the efficacy of non-invasive oocyte quality assessment techniques, a single oocyte culture system applicable routinely can meet several other objectives. Firstly, with single oocyte culture system embryos can be traced back to their original follicle (Oyamada and Fukui, 2004). From a scientific point of view, tracking individual oocytes with a specific known physiological background will greatly

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contribute to our knowledge on folliculogenesis and oogenesis. Secondly, a growing interest in oocyte and embryo metabolism, often characterized by variations in gene expression patterns, increases the need for individual production of embryos (Carolan *et al.*, 1996; Hagemann *et al.*, 1998; Fukui *et al.*, 2000; Krisher, 2004; Sturmey *et al.,* 2008). Thirdly, when IVP is used for screening purposes, for instance in toxicology studies, single oocyte culture could reduce the sample size. In this case, each single embryo can be considered as an independent statistical experimental unit, and no group effects have to be taken into account. In addition, single culture conditions are needed to avoid adherence of oocytes from which the zona pellucida had been removed (Vajta *et al.*, 2000). Such a system is also valuable to culture oocytes and embryos resulting from laborious manipulation methods, such as nuclear transfer and genotyping, where individual identification of oocytes is highly desired (Mizushima and Fukui, 2001; Pereira *et al.*, 2005). Also, for commercial reasons, a routine and reliable single oocyte culture system will be beneficial. Often, only one or a few oocytes can be obtained from a specific (bovine) donor following ovum pick-up (Bols, 2005), and these donors are often of high genetic value or belong to endangered species (Carolan *et al.*, 1996; O'Doherty *et al.*, 1997). Last but not least, individual bovine IVP can be a promising model for studying certain aspects of human infertility (Campbell *et al.*, 2003), particularly because in human assisted reproductive techniques (ART) oocytes and embryos are routinely cultured singly.

Hurdles to overcome when designing a bovine 'single oocyte' culture system

In cattle, embryo development in the female tract occurs usually individually. In this respect, routine bovine IVP systems are not representative of the physiological situation, as oocytes are cultured in groups in close contact with each other. Attempts to culture oocytes singly often have failed, or at least yielded disappointing results (Paria and Dey, 1990; Ferry *et al.*, 1994; Donnay *et al.*, 1997; O'Doherty *et al.*, 1997; Nagao *et al.*, 1998; Palasz and Thundathil, 1998; Hendriksen *et al.*, 1999; Jewgenow *et al.*, 1999; Yuan *et al.*, 2000; Fujita *et al.*, 2006; Feng *et al.*, 2007). Both developmental competence and embryo quality were affected, as shown by embryos with a lower cell number (Pereira *et al.*, 2005), a relatively smaller inner cell mass (Ahern and Gardner, 1998), low hatching rates (Carolan *et al.*, 1996; Hendriksen *et al.*, 1999; Larson and Kubisch, 1999; Yuan *et al.*, 2000; Goovaerts *et al.*, 2009) and low cryotolerance (Pereira *et al.*, 2005). Only a few groups achieved similar, or even higher blastocyst rates following single as compared to group culture (Hazeleger *et al.*, 1995; Carolan *et al.*, 1996; Vajta *et al.*, 2000; Han *et al.*, 2006), although embryo quality was not always satisfactory. Developmental success of group-cultured embryos can mainly be attributed to autocrine and paracrine communication between oocytes and/or embryos. The important growth factors that enhance *in vitro* development include insulin like growth factor I and II (IGF-I, IGF-II), transforming growth factor α and β (TGF-α, TGF-β), interferon τ (IFN-τ), epidermal growth factor (EGF), platelet-activated factor (PAF) and platelet derived growth factor (PDGF) (Paria and Dey, 1990; Thibodeaux *et al.*, 1995; Lim and Hansel, 1996; O'Neill, 1997). The concentration of several embryo-derived stimulatory factors seems to be crucial. Gopichandran *et al.* (2006) found that the distance of 165 µm between zygotes was ideal to fully enhance blastocyst and hatching rates, embryo cell number and carbohydrate metabolism. Low zygote numbers per drop and/or a lower embryo/medium ratio apparently influences the development in a negative way (Hoelker *et al.*, 2009). Single oocyte culture seems to be compromised by the lack of interaction with other oocytes/embryos. To increase the efficacy of individual development, the requirements of single developing oocytes need to be reconsidered; meanwhile, these findings will initiate the discovery of new oocyte quality parameters.

The development of a bovine 'single oocyte' culture system

To counteract the low levels of growth factors, standard culture media have been supplemented with a variety of growth factors that are tested during the different steps of (single embryo) *in vitro* production. While a detailed description of all culture media used is beyond the scope of this review, one of the most controversial topics is the use of serum. According to Carolan *et al.* (1996), the use of serum during individual maturation is detrimental, whereas others did not report this negative effect (Hagemann *et al.*, 1998). When single culture is performed in serum-free medium, the addition of amino acids and vitamins positively affected the development of flushed sheep zygotes (Gardner *et al.*, 1994). Whereas Li *et al.* (2006) enhanced single bovine embryo development by adding amino acids to a chemically defined culture medium, Mizushima and Fukui (2001) added hypotaurine and β-mercaptoethanol to the maturation medium of single oocytes leading to significantly higher fertilization rates and decreased polyspermy, as compared to the use of hypotaurine alone. Adding either β-mercaptoethanol or both however, did not cause significant differences in cleavage rates, but tended to improve blastocyst development compared to the control medium without additions. The addition of EGF to a group-IVP system did not affect blastocyst rate, whereas it did increase the number of blastocysts in the individual-IVP system (Oyamada *et al.*, 2004). Adding glutathione during single oocyte IVF significantly increased the proportion of normal fertilization and again decreased polyspermy

(Fukui *et al.*, 2000). Lim and Hansel (1996) demonstrated the positive effects of the addition of platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF-β_1 and β_2) on the developmental capacity of singly cultured 8-cell embryos. Those authors found that arachidonic acid, β-mercaptoethanol and glutathione stimulated the subsequent development of 8-cell embryos in the presence of PDGF and TGF-β_1 and β_2.

Alternatively to medium supplementation, somatic cells can be added to the zygote culture system to enrich environment conditions (Konishi *et al*., 1996). Using this procedure, individual oocytes were co-cultured with bovine oviductal epithelial cells (BOEC; Blondin *et al.*, 1997), Buffalo rat liver cells (BRL), cumulus cells (Donnay *et al.*, 1997) or granulosa cells (O'Doherty *et al.*, 1997). Goovaerts *et al*. (2009) intensively tested the use of cumulus cells in a co-culture system. In a first experiment, they observed an increase in blastocyst rate from 2.9 up to 21.8% when adding cumulus cells to the *in vitro* culture. Later work confirmed these results and also defined the blastocyst quality in terms of cell number, apoptotic cell ratio and gene-expression of 10 embryo-quality related genes (Goovaerts *et al*., 2011). They concluded that the quality of singly produced embryos is similar to their group-cultured counterparts in all aspects, except for the expression of GPX1. This gene is involved in detoxification and mtDNA protection to oxidative stress, and was downregulated in singly produced embryos. This result re-opens the debate on the use of co-culture systems with somatic cells in IVP systems. Although it undoubtedly increases the amount of non-defined factors in the production system, the added cells clearly support embryo development and improve embryo quality by the secretion of embryotrophic factors and neutralizing embryo-toxic components (de Wit and Kruip, 2001; Orsi and Reischl, 2007, Goovaerts *et al*., 2011). Equally undefined but as an alternative for the cumulus cell co-culture to achieve paracrine support and stimulation of singly cultured oocytes is the use of conditioned media. Cumulus-oocyte complexes or somatic cells are previously cultured and the supernatant is subsequently used as culture medium (Fujita *et al.*, 2006). However, this method did not result in (consistently) high blastocyst rates (Goovaerts *et al*., 2009).

The gas atmosphere surrounding the oocytes and embryos during *in vitro* development also changes the microenvironment, and thus influences embryo metabolism (Krisher, 2004) due to a link with the production of reactive oxygen species (ROS). Hagemann *et al.* (1998) did not find a significant difference in developmental rates between 7 and 20% O_2 during maturation or fertilization of single bovine oocytes, whereas Oyamada *et al.* (2004) reported higher cleavage and blastocyst rates following maturation at 20% O_2 compared to 5%. Individual pig embryo development improved in an atmosphere of 5% O_2 and 5% CO_2 (Berthelot *et al.*, 1996).

To create equilibrium among the autocrine factor dilution, the accumulation of toxic metabolites and the availability of nutrients during culture, a medium replacement schedule can be applied (Ferry *et al.*, 1994). However, based on available literature, it was never tested for individual culture. In addition, droplet size can be adapted, as substrate depletion and build-up of toxic substances can occur in micro-drops. While single embryo culture was compromised in droplet sizes smaller than 10 µl (Carolan *et al.*, 1996; O'Doherty *et al.*, 1997; Nagao *et al.*, 1998), single fertilization in 10 µl was shown to decrease polyspermia but also the penetration rate, compared to 25, 50 or 100 µl droplets (Fukui *et al.*, 2000).

To mimic the *in vivo* situation, where the embryo is embedded in a small amount of reproductive tract secretions containing growth factors at high concentrations, but keep embryos individually identifiable at the same time, Vajta *et al.* (2000) designed the "well of the well" system (WOW) for *in vitro* culture. About 10 to 15 V-shaped small wells were created into the bottom of a standard 4-well plate with a heated polished steel rod. The four large wells were filled with 500 µl of culture medium and covered with oil after which a zygote was cultured in each V-shaped well. While the volume of each of the smaller wells was only 0.04 µl, limiting the dilution of autocrine factors, the large amount of medium above these wells was assumed to provide nutrients and to dilute toxic metabolites. This culture system resulted in higher blastocyst rates than conventional group culture or single culture in 20 µl droplets, with a blastocyst cell number similar to that of group cultured embryos. This system allows individual tracking of single oocytes and zygotes, but the zygotes can communicate through the supernatant medium and exchange growth factors with neighboring embryos which can be undesirable if one is interested in individual developmental characteristics of a specific embryo. Recently, Hoelker *et al.* (2009) showed similar blastocyst rates, differential cell counts and apoptotic indexes in the WOW culture system with 16 zygotes in shared medium, and control group culture with 50 zygotes per droplet. In contrast, culture of 16 zygotes in a standard droplet decreased the outcome compared to those cultured in the WOW system. In human assisted reproduction, pregnancy was efficiently established after using embryos cultured in the WOW system (Vajta *et al.*, 2008). A variation of this "apart-together" culture system, allowing both stimulation and individual identification, is to culture one zygote separated from a group of embryos by a mesh barrier (Spindler *et al.*, 2006; Somfai *et al.*, 2010). Han *et al.* (2006) developed a WOW comparable system, the well-in-drop (WID) culture system, for single goat oocytes, whereas others (Dode *et al.*, 2003; Pereira *et al.*, 2005; Spindler *et al.*, 2006) slightly modified the WOW

system by covering each individual small well with 20 µl of medium and using co-culture with cumulus cells in a high O_2 atmosphere. Under these conditions, no shared medium over-layer is used, but lipophilic factors can be absorbed and diffused through the oil into other medium droplets. No higher blastocyst rates were reported, as compared to an individual 20µl standard droplet culture system, but cell numbers of modified WOW cultured embryos were higher. A recent study (Matoba *et al.*, 2010) compared three 'apart-together' systems: WOW, adhesive Cell-Tak and a polyester mesh, resulting in comparable developmental blastocyst outcomes but with a preference for the latter in terms of the easier preparation and use.

More recently, novel physical environments for embryo culture were designed, such as microfluidic devices (Smith and Takayama, 2007; Wheeler *et al.*, 2007; Smith *et al.,* 2012) and glass oviducts or micro-tube culture (Thouas *et al.*, 2003; Roh *et al.*, 2008). These systems permit, in contrast to micro drops under oil, the use of very small volumes of medium without oil overlay. The microfluidic devices add a dynamic culture environment with a medium flow that washes toxic metabolites away, but it simultaneously dilutes autocrine/paracrine factors. These huge changes in physical environment are likely to affect individual embryo development very differently, and might be promising for individual production of bovine embryos, but have not yet been tested under routine conditions. Some research groups used single oocyte culture as a tool to link follicle environment and cumulus-oocyte complex characteristics to developmental competence of the oocyte involved (Araki *et al.*, 1998; Hagemann *et al.*, 1999; Jewgenow *et al.*, 1999; Han *et al.*, 2006).

Toxicity screening as a 'ready to use' application for bovine 'single embryo' culture

Because of decreasing interest in IVP for cattle production systems, more attention has been given to the possible role of the bovine *in vitro* embryo for research purposes. Given that the embryo *in vitro* culture is a fairly well defined procedure that can easily produce living specimen composed of pluripotent cells, makes it a possible target to study, for example, the impact of toxic substances at the embryonic level. While an overview on the possible influences of environmental toxicants on gamete and embryo quality is far beyond the scope of this mini-review, it has repeatedly been shown that, for example, endocrine disrupting compounds (EDCs) have a negative influence on oocyte and subsequent embryo quality, even at the follicular level (Pocar *et al*., 2001, 2006; Brevini *et al.*, 2004; Petro *et al*., 2012). However, rendering the single oocyte culture system ready for toxicity screening purposes requires again a few specific modifications. First of all, one has to check and define the possible historical contamination of the IVP system that is in use for single embryo production. We could not detect significant amounts of endocrine disrupting compounds in tissues and body fluids, including follicular fluid, when sampling oocyte donors following slaughter (Petro *et al.*, 2010). A second hurdle to overcome is the elimination of the use of oil for single embryo production, because most of the endocrine disrupting compounds are lipid soluble. Thus, oil use could bias toxicity screening protocols prescribing the addition of EDCs to the culture environment. Very recently, we succeeded in developing oil-free, semi-defined cultured conditions for single IVP (Goovaerts *et al.*, 2012). The next logical step will be the addition of EDCs to the single oocyte culture system during a short time frame and study acute toxic effects by defining mRNA production levels in exposed and controlled embryos.

Conclusions

It is clear that individual culture of (bovine) oocytes to single embryos is still compromised as compared to group culture. However, similar blastocyst rates are within hands and the embryo quality of singly and group cultured embryos is comparable. The method of choice depends on the indication for single oocyte culture to be used. A chemically defined medium without co-culture or oil seems essential to study oocyte and embryo metabolism or when the single embryo will be used as a tool in toxicity screening. If applied in a commercial setting, namely to produce the highest possible number of good quality embryos from a limited number of oocytes, the use of serum and co-culture systems will be beneficial. No matter which setting is used, single embryo production will help to unravel the complex interactions between the oocyte and somatic cells without the influence of other oocytes.

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