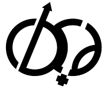

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In vitro culture systems: how far are we from optimal conditions?

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Abstract

Over the past decades *in vitro* production (IVP) of bovine embryos has been significantly improved. Nevertheless, embryos generated *in vitro* still differ from their *in vivo* produced 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.

Keywords: cattle, embryo, *in vitro* production.

Introduction

The birth of the first IVF calf derived from an *in vivo* matured oocyte in 1981 (Brackett *et al*., 1982) and the discovery of heparin as capacitating agent for bull sperm in 1986 (Parrish *et al*., 1986) were the two key events starting an era of intense research resulting in efficient *in vitro* production (IVP) systems for preimplantation embryos including *in vitro* maturation (IVM) of the oocyte to the metaphase II, *in vitro* fertilization (IVF), and subsequent *in vitro* culture (IVC) of embryos to the blastocyst stage. The first calves produced entirely from IVM-IVF-IVC were born in 1987 (Fukuda *et al*., 1990).

Another milestone was the development of ultrasound-guided transvaginal oocyte aspiration in humans and the adoption in the bovine in 1988 (Pieterse *et al*., 1988). The current technology of OPU/IVP harvesting immature oocytes form living cows can routinely be performed twice a week for an extended period of time without any long-term detrimental effects on the donor's cow fertility (Chastant-Maillard *et al*., 2003). Attempts were undertaken to combine OPU with colour Doppler ultrasonography which is a useful, noninvasive technique for evaluating ovarian vascular function, allowing a visual observation of the blood flow in a delimited area in the wall of preovulatory follicles (Brännström *et al*., 1998). Blood flow determinations of individual preovulatory follicles prior to follicular aspiration for IVF therapy provide an important insight on the intrafollicular environment and may predict the developmental competence of the corresponding oocyte (Coulam *et al*., 1999; Huey *et al*., 1999). In cattle, it has been shown that the time interval between the individual OPU sessions had an effect on the quality of oocyte and embryos at the molecular level, whereas differences in the perifollicular blood flow did not (Hanstedt *et al*., 2010). An increase in the blood supply to individual follicles appears to be associated with follicular growth rates, while a reduction seems to be closely related to follicular atresia (Acosta *et al*., 2003; Acosta 2007). Taken together, OPU can be considered a mature technique and no major improvements should be expected in the technology and its results in the near future. At present the application of IVP combined with ovum pick up (OPU) from valuable donors is increasing (again) due to developing breeding strategies based on genomic selection using SNP (single nucleotide polymorphism) chips. Depending on the chip used, thousands of these SNPs can be analyzed even in a biopsy taken from an embryo. This technology is now reaching routine usage for genomic selection (GS) in cattle (Ponsart *et al*., 2013).

With regard to IVP efficiency, approximately 80-90% of immature bovine oocytes undergo nuclear maturation *in vitro*, about 80% undergo fertilization, 30-40% develop to the blastocyst stage, and around 50% of the transferred embryos establish and maintain a pregnancy (Wrenzycki *et al*., 2007; Galli *et al*., 2014; Lonergan *et al*., 2016).

IVM

Cumulus-oocyte-complexes (COC) collected from ovaries of slaughtered or euthanized animals or from living animals via ovum pick-up (OPU) require *in vitro* maturation (IVM) as they are arrested at the germinal vesicle (GV) stage. Maturation involves a series of events that begin in fetal life with the initiation of meiosis. At birth, the oocytes are arrested at the diplotene stage (germinal vesicle stage, GV). After puberty when they are exposed to preovulatory surges of LH and FSH they proceed with meiosis and are arrested again at the metaphase II, the stage at which they are ovulated (Monniaux *et al*., 2014). In addition, optimal conditions for cumulus cells surrounding the oocyte need to be considered as there is a complex bi-directional communication between these two cell types (Gilchrist, 2011; Monniaux, 2016).

Proper maturation of the oocyte to metaphase II is a prerequisite for fertilization and pre-implantation development. It is possible to achieve blastocyst rates of up to 70% if *in vivo* matured oocytes are used. In contrast, if oocytes are matured *in vitro*, blastocyst rates are only half that of those matured *in vivo*. This rather limited success may be attributed to the heterogeneous population of oocytes which are normally retrieved from follicles of 3-8 mm rather than from preovulatory follicles. In contrast to the *in vivo* ovulated oocyte, these oocytes lack development up to the preovulatory stage

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and are matured *in vitro*. Therefore, much effort has been devoted to the establishment of noninvasive and non-perturbing means for selecting the most competent oocytes (Fair, 2010; Krisher, 2013; Wrenzycki and Stinshoff, 2013).

IVM of immature oocytes occurs by a different mechanism from that of *in vivo* matured oocytes. IVM is initiated immediately following the removal of the immature oocyte from small antral follicles, and such oocytes may have neither the time nor the correct environment to complete the necessary changes required for subsequent successful development (Krisher, 2013; Wrenzycki and Stinshoff, 2013; Lonergan and Fair, 2016).

Recently, the so-called simulated physiological oocyte maturation (SPOM) system has been introduced (Albuz *et al*., 2010). It prevented spontaneous resumption of meiosis after mechanical oocyte retrieval and thereby improved *in vitro* embryo development. However, due to the fact that similar outcomes were not easily to achieve, a revised version has been reported (Gilchrist *et al*., 2015). At the moment, most laboratories practicing IVM of cattle oocytes use a relatively simple oocyte maturation system.

IVF

IVF is a complex procedure whose success requires appropriate oocyte maturation, sperm selection, sperm capacitation and IVF media.

Semen samples contain a heterogeneous population of sperm cells. *In vivo*, the sperm cells are thought to be selected by various mechanisms within the female reproductive tract, with the result that the small number of spermatozoa found near to the oocyte are typically those best able to penetrate the zona pellucida and fertilize the oocyte. When using IVF, however, these natural selection mechanisms are circumvented. The most common method for preparing spermatozoa for IVF is by centrifugation them through a concentration gradient, such as a 45% Percoll mixture layered on a 90% solution.

A variant of colloid centrifugation using only one layer of colloid (in which case there is no gradient) has been developed. 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 (Thys *et al*., 2009; Goodla *et al*., 2014; Morrell *et al*., 2014; Gloria *et al*., 2016). An alternative method is the swim-up procedure. The disadvantages of swim-up are that it takes approximately 45-60 min to do and only 10-20% of the spermatozoa in the sample are recovered. For colloid centrifugation, only 25 min preparation time is needed (including the centrifugation) and a recovery rate of >50% is commonly achieved (Thys *et al*., 2009), although this does depend on the sperm quality of the original sample.

Once IVM is complete, oocytes are ready to be fertilized. This involves the coincubation of oocytes with sperm cells. Most laboratories allow for 18-19 h of coincubation. The changes a sperm cell has to go through before it can fertilize an oocyte are summarized under the term capacitation. Media have been developed to support this process, e.g. TALP medium. As mentioned earlier, the primary capacitation agent is heparin. The majority of semen used for IVF is frozen-thawed. The most common final sperm concentration used in the IVF drop is 1 x 10^6 sperm/ml.

IVC

IVC of bovine embryos is the last step in the IVP procedure and involves approximately 6 days of culture from the presumptive zygote onwards. The most common media for culturing bovine embryos are variations of the original synthetic oviduct fluid (SOF) medium (Tervit *et al*., 1972). SOF is now part of most routine bovine IVP systems with/without serum. Embryos are cultured in only one medium throughout the entire time or a sequential system in which the medium formulation changes at certain time points in the culture period. These sequential media try to mimic the physiological changes that embryos encounter *in vivo* when they move down the oviducts and into the uterus. Parameters which vary from lab to lab are diverse, e.g. the volume of medium and the atmosphere in the incubator.

In vitro culture (IVC) conditions have been enhanced in the last years, mainly by adjustment of media formulations. However, while over 30% blastocyst formation could be achieved in most culture systems, it soon became obvious that quantity did not always match quality (Wrenzycki *et al*., 2005; 2007 Lonergan *et al*., 2006;) 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 abnormally advanced embryonic and fetal growth, altered gene expression patterns, and high perinatal losses (Young *et al*., 1998; 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 Wagtendonk-de Leeuw *et al*., 2000). Such observations highlight the importance of the post-fertilization culture environment for the quality of the resulting blastocyst. However, the existence of diverse embryo culture media and methods makes it very challenging to define the optimal components of embryo culture media.

The success of an IVP laboratory may stem not only from improvements of the IVC per se, but from the entire IVP system (Gardner, 2008; Baltz, 2012; Leese, 2012). The latter includes: incubation conditions, gas phase, culture media, oil overlay, plastic ware, and embryo density and the volume of the medium. In addition, the skills of the staff involved in the entire process have to be considered as part of the system.

In general, IVP can be considered to be at an advanced stage of progress. However, an aspect that may change in the future is automation and miniaturization of the IVP process by better mimicking the *in vivo* environment, e.g. using microfluidics (Wheeler *et al*., 2007) or an encapsulation technology (Blockeel *et al*., 2009) to obtain IVP embryos of similar

quality as the *in vivo* ones.

Quality assessment of 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. Morphology and the proportion developing to the blastocyst stage are used as criteria to assess developmental competence. Evaluation of embryo morphology remains the method of choice for selection of viable embryos prior to transfer. It is the most practical and clinically useful approach to assess of embryo viability (Van Soom *et al*., 2003). A bovine embryo grading system developed previously (Lindner and Wright, 1983) is, with minor modifications, still widely applied in this field (Hasler, 2001), listed in the IETS Manual. However, sometimes embryo morphology alone is not accurate enough to act as the sole criterion for the prediction of embryo developmental potential *in vivo.* Better non-invasive markers and improved techniques are required. These techniques can provide more valuable information on embryo viability. For examples, measurement of oxygen consumption using the nanorespirometer (Lopes *et al*., 2007) as well as amino acid profiling (Sturmey *et al*., 2010) can be employed to predict developmental competence and embryo viability. Although non-invasive approaches are improving, invasive ones have been extremely helpful in finding candidate genes to determine embryo quality (Wrenzycki *et al*., 2007; Rizos *et al*., 2008; Graf *et al*., 2014).

Conclusion

In vitro production (IVP) of bovine embryos follows a well-developed procedure that is commercially available for this species. However, despite all the improvements in oocyte and embryo culture, at best only 30-35% of immature bovine COC develop to the blastocyst stage which might represent a reasonable efficiency. But the *in vivo* situation still cannot be mimicked sufficiently well. The quality of the embryos produced is still impaired in comparison with their *in vivo* counterparts. This suggests that there are still improvements to be made in increasing oocyte and embryo developmental competence. More basic research is needed on molecular mechanisms e.g. epigenetic reprogramming during early embryonic development as well as detailed studies on the composition and interactions of culture media. By altering the conditions of oocyte maturation and embryo culture respectively to mirror more closely that which occurs *in vivo*, it may be possible to produce not only more blastocyst stage embryos, but more importantly, blastocysts of better quality.

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