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High throughput non-invasive oocyte quality assessment: the search continues

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Abstract

Non-invasive oocyte quality assessment remains a major challenge of routine bovine *in vitro* embryo production (IVP). There is a major need for techniques allowing early selection of developmentally competent oocytes on the basis of a simple, quick, economic and feasible protocol. The availability of such a technique would clearly increase IVP efficiency since only competent oocytes would be used, maximising blastocyst yield by ameliorating culture conditions. This mini-review summarizes briefly the currently available techniques that allow high throughput non-invasive oocyte quality assessment and indicates their possibilities and limitations.

Keywords: BCB, bovine oocyte quality, *in vitro* embryo production (IVP), non-invasive oocyte quality assessment, single oocyte culture.

Introduction

For several decades now, puncture of immature ovarian follicles has been used to retrieve oocytes for *in vitro* embryo production (IVP). Various excellent updates reviewing IVP and embryo transfer in domestic animals indicate that the availability of 'good' quality oocytes is the pre-requisite for high blastocyst rates (Hasler *et al*., 1998; Galli *et al*., 2001; Merton *et al*., 2003). Oocytes for IVP can be retrieved from different sources: postmortem oocyte recovery from slaughterhouse ovaries as well as transvaginal ultrasound-guided follicle aspiration (OPU) in living donors. Many important factors play a role in determining the final intrinsic developmental capacity and quality of the oocytes obtained. Most of these are donor-related, as extensively reviewed by Merton *et al*. (2003): stage of the estrous cycle at the time of retrieval, follicular status related to oocyte growth and final maturation, donor condition and breed, hormonal stimulation prior to OPU and the number of oocyte collections within a specific time span. All of these can be considered as 'biological factors', acting separately or in combination for a specific donor. In parallel, there is a group of more 'technical factors' (Bols, 2005), intrinsically related to the retrieval procedure: follicle visualization, needles and aspiration vacuum used and cumulus oocyte complex (COC) processing immediately before IVP. Irrespective of which factor can be defined as crucial for a certain donor, there is a general agreement that the importance of an intact cumulus cell investment for oocyte maturation and *in vitro* development cannot be overestimated (Zhang *et al*., 1995; Konishi *et al*., 1996, Boni *et al*., 2002). While the effects of biological factors may vary widely among donors, the method of retrieval clearly has a more defined impact on COC morphology, and therefore also on subsequent developmental capacity *in vitro* (Takagi *et al*., 1992; Hamano *et al*., 1993; Bols *et al*., 1996, 1997).

Given the important role of the cumulus cells (for review see Tanghe *et al*., 2002), immature bovine oocytes have always been divided into different quality categories, based upon light microscopic evaluation of the compactness of the cumulus cell layers and transparency of the cytoplasm (de Loos *et al*., 1989; Hazeleger *et al*., 1995). After all, it has been sufficiently documented that IVP with oocytes with an incomplete or damaged cumulus investment results in a significantly lower blastocyst outcome, compared to culture of COCs with a complete, dense cumulus (Shiowa *et al*., 1988; Merton *et al*., 2003). Close contact between cumulus cells and the ooplasm is established through cumulus cell process endings (CCPEs), which penetrate the cortex and make gap junctions with the oolemma in the best quality oocytes (de Loos *et al*., 1991), whereas this is not the case in their low quality counterparts. On the other hand, quality assessment of the cumulus cell layer, on the basis of morphological characteristics, has to be used with great care. Blondin and Sirard (1995) showed that oocytes with signs of beginning expansion in the outer cumulus cell layers and a slightly granulated ooplasm developed significantly more past the 16-cell stage than others. Boni *et al*. (2002) showed higher embryo production efficiency with grade B (dark and compact cumulus cytoplasm) COC quality.

Limits to the determination of 'ad hoc' quality of freshly retrieved oocytes

Once the oocytes are aspirated, retrieved and grouped for *in vitro* maturation, the immediate link between the individual oocyte and its specific follicular environment and physiological history is lost. Although this link with the originating follicle is undoubtedly extremely important for subsequent oocyte development (Vassena *et al*., 2003), the only non-invasive quality assessment parameter left at this stage is indeed COC

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morphology. Tracking individual oocytes and studying factors that influence their quality from this point on will certainly benefit from two important techniques: individual or 'single' oocyte culture conditions to follow oocytes 'over time' and non-invasive (immature) oocyte quality assessment techniques to monitor oocytes 'quality-wise'. Because in all routine IVP systems, group culture of COCs is a prerequisite to achieve acceptable blastocyst rates, current protocols do not permit to trace back the individual oocyte to the follicle it originates from. With all due respect for the excellent work of many researchers worldwide, using invasive technology to unravel factors determining oocyte quality (beyond the scope of this paper, but excellently reviewed by Krisher, 2004), there is an urgent need for additional non-invasive quality assessment procedures. Ideally, individual oocytes should survive quality assessment and re-enter into the IVP system to develop into a blastocyst and thereby eventually confirm the outcome of the initial quality assessment. This paper offers only a brief summary on the scarce high throughput, non-invasive oocyte quality assessment techniques that are currently available.

The need for high throughput, non-invasive oocyte quality parameters

As suggested above, the ideal single oocyte culture protocol should permit 'built-in' oocyte quality assessment by which the oocyte is subjected to quality tests and immediately returned into, or better, not even removed from the IVP system. If this could be performed on a single oocyte, the retrieved information can be traced back to the oocyte's origin and correlated to the (blastocyst) outcome of the IVP system. Clearly, the ultimate quality assessment tool is the outcome of the IVP procedure itself since only 'good quality' oocytes have the ability to further develop to the blastocyst stage *in vitro* (Sirard *et al*., 2006). Alternative non-invasive oocyte quality assessment techniques need to meet several specific criteria to be applicable in routine IVP systems. While the major goal for a commercial IVP set up would be to select those oocytes with the highest developmental potential, preferentially before maturation, low quality oocytes could be discarded from the production system in the mean time. This will undoubtedly benefit the development of the remaining oocytes as well as the economics of the procedure. The ideal technique to assess quality parameters should be easy to perform, economically sound, quick, allow a high throughput of COCs, have an acceptable reliability and above all, be non-invasive, so that the oocyte's developmental capacity is not hampered by quality assessment.

Current availability of non-invasive oocyte quality assessment parameters

As stated earlier, although very valuable (Fair, 2003, 2010), keeping the link between the oocyte and its originating follicle is not an option in a commercial bovine IVP environment. When assessing oocyte quality upon retrieval, immature bovine oocytes for IVP are usually selected on the basis of cumulus investment morphology and homogeneity of the cytoplasm (de Loos *et al*., 1988; Hazeleger *et al*., 1995). In the mean time, many studies have tried to link these morphological aspects to the oocyte's developmental capacity, with variable success (review Bols, 2005). Indeed, even with the best quality COCs, based on their morphology, only around 35-45% blastocyst can be produced with routine IVP protocols, which strongly suggests a need for additional/alternative oocyte quality parameters.

The use of a vital blue dye, brilliant cresyl blue (BCB), to select oocytes suitable for IVP, is substantially documented in the literature. One of the first reports describes the use of BCB for the selection of pig oocytes for IVM and IVF (Ericsson *et al*., 1993). The intensity of the BCB staining is basically a measure for the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme that is synthesized by the oocyte during oogenesis as part of the pentose phosphate cycle (Alm *et al*., 2005). It is particularly active in growing oocytes with a clear decrease in activity when the oocyte finishes its growth phase (Mangia and Epstein, 1975). Brilliant cresyl blue is a blue dye that is reduced to a colorless substance through the action of G6PD. Fully-grown oocytes show a decreased G6PD activity, meaning that their cytoplasm will remain blue following the uptake of BCB. This specific characteristic makes BCB an indicator for G6PD activity and therefore indirectly indicates the growth stage of the oocyte. Rodríguez-González *et al*. (2002) used BCB to select more competent pre-pubertal goat oocytes for *in vitro* embryo production. They exposed oocytes to BCB diluted in PBS and subsequently classified them according to the color of their cytoplasm: oocytes with a blue cytoplasm or 'grown' oocytes (BCB+) and oocytes with a colorless cytoplasm or 'growing' oocytes (BCB-). They showed that BCB+ oocytes were significantly larger than BCB- ones, while a higher proportion of BCB+ oocytes reached the MII stage compared to BCB- and non-treated oocytes. These results were recently confirmed for pre-pubertal BCB+ sheep oocytes, which were larger and more competent to develop into blastocysts with a higher number of cells (Catalá *et al.,* 2011). Accordingly, several reports stress the importance of oocyte diameter in relation to developmental competence (Lonergan *et al.*, 1994; Fair *et al*., 1995; for review see Fair, 2003, 2010; Vandaele *et al*., 2007). In addition, the proportion of embryos developing beyond the 8-cell stage and the number of morulae/blastocysts was higher for BCB+ oocytes. Surprisingly, around 50% of the oocytes graded as best quality, on the basis of routine morphological selection criteria, appeared to

be BCB-. In other words, these oocytes passed first selection on morphology successfully, but should be excluded from IVP on the basis of the BCB test.

Similarly, Pujol *et al*. (2004) used the BCB test to assess the developmental competence of heifer oocytes. While using a slightly modified oocyte quality grading system, they showed that nearly 79% of the morphologically classified grade 1 oocytes were BCB+ and confirmed that BCB+ oocytes were larger and more competent for IVP than control heifer oocytes. Alm *et al*. (2005) reported on the use of BCB to increase the efficiency of *in vitro* blastocyst production by oocyte selection before maturation. They showed a significantly higher rate of maturation to metaphase II for control and BCB+ oocytes compared to BCB- oocytes. Interestingly, they only used COCs with a compact cumulus investment, retrieved by slicing slaughterhouse ovaries, meaning that they combined morphological characteristics and the BCB test in their oocyte selection protocol. About 58% of the oocytes from COCs retrieved turned out to be BCB+, which is on the low side when compared to 75.6% BCB+ oocytes in pigs, reported by Roca *et al*. (1998). The authors attribute this lower percentage of BCB+ oocytes to their oocyte retrieval technique, because slicing most probably leads to the collection of more growing oocytes, collected from smaller follicles. Although this 'double' COC screening resulted in a substantial increase in the number of blastocysts in IVP with BCB+ oocytes, compared to controls (34.1 *vs*. 19.2% respectively), the final absolute number of blastocyst did not increase, compared to other reports in literature, which stresses the underlying importance of the oocyte retrieval technique (slicing *vs.* aspiration) and the IVP culture system. Ishizika *et al.* (2009) reported that matured porcine BCB+ oocytes have higher meiotic competence (MII spindle) and intra-oocyte glutathione levels, indicating a better cytoplasmatic maturation. Egerszegi *et al*. (2010) confirmed these results, stating that porcine BCB+ oocytes were characterized by the appearance of fibrillated chromatin filaments in their germinal vesicles, whereas BCB- oocytes mainly contained condensed stages of chromatin. After 22 h of IVM, BCB+ oocytes showed a prominent chromatin configuration of metaphase I and after 44 h, the majority developed a MII nuclear configuration, in contrast to BCB- oocytes. At the beginning of IVM, BCB+ oocytes displayed high mitochondrial activity and a homogenous distribution of mitochondria as well as more aggregated clusters, opposite to BCB- oocytes. Torner *et al.* (2008) tried to identify molecular and subcellular differences between BCB+ and BCB- oocytes by using Western Blot analysis for the phosphorylation pattern of protein kinases, a cDNA microarray for gene expression profiles, and fluorescence labeling and photometric measurement for chromatin configuration of the nucleus and the mitochondrial activity of the oocytes. This broad approach revealed molecular organizational variations in oocytes with different G6PDH activity, such as a lower mitochondrial activity in BCB+ oocytes. In contrast, Catalá *et al*. (2011) reported a higher mitochondrial activity in BCB+ oocyte from pre-pubertal sheep after IVM, while maturation-promoting factor (MPF) was more active at the metaphase II stage in BCB+ as compared to BCB- oocytes. Brilliant cresyl blue stained ovine oocytes showed significantly higher maturation rates, a subsequently higher number of cleaved embryos and significantly more blastocysts when compared to BCB- oocytes (Mohammadi-Sangchesmeh *et al.*, 2012). The same group reported similar results for BCB+ equine oocytes, which turned out to have a larger diameter and a higher maturation rate and turned into blastocysts at a higher rate as compared to their BCB- counterparts (Mohammadi-Sangchesmeh *et al.*, 2011). All of this was substantiated by a different gene expression pattern for BCB+ and BCB- oocytes. Other studies (Opiela *et al.,* 2008; Vandaele, 2008) failed to demonstrate a correlation between oocyte G6PDH activity, as assessed via BCB, and the expression of apoptotic genes or the apoptotic cell ratio in subsequently developed embryos.

Fortunately, authors report no alterations in fertilization and cleavage rates following the use of BCB, which is a reassuring outcome when considering the BCB test for a routine application. While additional reports reiterate the value of the BCB test for the selection of developmentally competent oocytes (Sugulle *et al*., 2006) related to bovine nuclear transfer procedures (Bhojwani *et al*., 2007) and for use with COCs collected by OPU (Tagawa *et al*., 2006), there are no indications of a routine application of this assessment method in bovine IVP. Moreover, no reproducible differences can be found in blastocyst rates obtained with morphologically selected control COCs and BCB+ selected oocytes. In addition, not one study reports blastocyst rates obtained with BCB+ oocytes higher than the 30-40% that can be obtained in routine IVP with morphologically selected oocytes. Also, the procedure requires 90 min incubation, making the technique time-consuming (personal observations), which in itself might affect the overall IVP efficiency (Vandaele, 2008).

Finally, as stated earlier, an enormous amount of knowledge has been generated by studying the interplay among the follicle as a whole, the cumulus cells and the enclosed oocyte (Tanghe *et al*., 2002; Fair 2003, 2010). Describing this link in detail is far beyond the scope of this paper. However, an interesting noninvasive approach to oocyte quality assessment is offered by the idea that knowledge on cumulus cell quality might tell us something about the developmental potential of the corresponding oocyte. Similarly to oocytes, we can distinguish morphological (noninvasive) as well as biochemical and cytological (invasive) parameters for cumulus cell quality

assessment. Whereas the first can be applied on a routine basis (Laurinčik *et al*., 1996), the latter most often involve a biopsy of the cumulus cells, to be as non-invasive as possible to the COC as a whole. While this procedure does not necessarily impair COC developmental competence, it is very laborious and not applicable to a routine IVP system. Moreover, while it is still uncertain if cumulus cell characteristics are good predictors for the oocytes' developmental competence (Han *et al*., 2006; Anguita *et al*., 2007), the developmental stage of the cumulus cells and the species studied will definitely play an additional role (McKenzie *et al*., 2004). Although it has clearly been demonstrated that the composition of follicular fluid can be a predictor of *in vitro* embryo development (Sinclair *et al*., 2008; Leroy *et al*., 2012), follicular fluid analysis is less practical in commercial OPU-IVP programs (Revelli *et al*., 2009). While excellent research worldwide has clearly demonstrated that the molecular approach towards oocyte quality assessment results in an enormous amount of exciting additional insights (for review: Patrizio *et al*., 2007), these techniques are not applicable in a high throughput system where hundreds of oocytes need to be processed in the shortest possible time span.

Conclusions

The amount of knowledge on factors that determine the oocytes' *in vitro* developmental competence is increasing at an enormous speed. Looking specifically at bovine routine IVP systems, which use immature oocytes, group culture is still preferable to achieve acceptable blastocyst rates. As a consequence, it remains difficult to follow an individual oocyte through the IVP procedure and draw conclusions on the developmental capacity of the original cumulus oocyte complex. Despite the fact that promising results are recently obtained with strictly single oocyte culture systems (Goovaerts *et al*., 2009, 2010 (review), 2011), studying the factors that determine the oocytes' developmental ability will still be hampered because most of the competence studies include parameters which can only be assessed by sacrificing the oocyte. Only by combining research efforts on the development of single oocyte culture protocols and a search for additional non-invasive oocyte quality assessment techniques, it will be possible to trace individually cultured oocytes through the IVP system and draw direct conclusions on factors that can predict oocyte developmental potential *in vitro*. Hopefully, this will ultimately lead to a better and early selection of oocytes for *in vitro* production with less expensive, yet more embryos of a better quality as a result.

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