Review

Noninvasive bovine oocyte quality assessment: possibilities of a single oocyte culture

I.G.F. Goovaerts*, J.L.M.R. Leroy, E.P.A. Jorssen, P.E.J. Bols

University of Antwerp, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, Laboratory of Veterinary Physiology, Universiteitsplein 1, Gebouw U, B-2610 Wilrijk, Belgium

Received 10 May 2010; received in revised form 21 June 2010; accepted 22 June 2010

Abstract

Although bovine embryos are routinely produced *in vitro* for several decades, there still exists a critical need for techniques to accurately predict the oocyte's developmental competence in a noninvasive way, before the *in vitro* embryo production procedure. In this review, several noninvasive methods to evaluate oocyte quality are discussed, such as morphological assessment of the cumulus oocyte complex and the use of brilliant cresyl blue. Because an individual oocyte and embryo culture method can possibly generate additional insights into the factors that determine oocyte quality, the second part of this review summarizes the state of the art of bovine single oocyte culture. The optimization of individual *in vitro* embryo production can obviously accelerate the quest for better noninvasive oocyte quality markers, because more information about the oocyte's requirements and intrinsic quality will be revealed. Although each step of *in vitro* culture has to be re-examined in light of the hampered production of single embryos, the reward at the end will be substantial. Individual scored oocytes will be traceable along the *in vitro* embryo production procedure and the final blastocyst outcome can be linked to the original oocyte quality and follicular environment without the bias caused by simultaneously developing embryos.

© 2010 Elsevier Inc. All rights reserved.

Keywords: Bovine oocyte; Individual *in vitro* embryo production; Single oocyte culture; Noninvasive oocyte quality assessment

Contents

1. Introduction	1510
2. The need for routine, noninvasive oocyte quality parameters	1510
2.1. Morphology	1511
2.2. Glucose-6-phosphate dehydrogenase	1511
2.3. Cumulus cell investigation	1512
2.4. Future perspectives	1513
3. The development of a routine single oocyte culture system	1513
3.1. Purposes	1514
3.2. Obstacles	1514
3.3. Medium supplementation	1514
3.4. Co-culture	1515

* Corresponding author. Tel.: + 32 3 265 23 95; fax: + 32 3 265 24 33.

E-mail address: Ilse.Goovaerts@ua.ac.be (I.G.F. Goovaerts).

3.5. Gas atmosphere	1515
3.6. Medium replacement and droplet size	1516
3.7. Physical environment and dynamic culture conditions	1516
4. Conclusions	1517
Acknowledgements	1518
References	1518

1. Introduction

For several decades now, puncture and aspiration of ovarian follicles has been used to retrieve oocytes for *in vitro* production (IVP) of bovine embryos. Various comprehensive updates reviewing IVP and embryo transfer (ET) in domestic animals indicate that the availability of “good” quality oocytes is a prerequisite for success [1–3]. Oocytes can be derived from different sources, including postmortem oocyte recovery from slaughterhouse ovaries as well as (repeated) transvaginal ultrasound-guided follicle aspiration (OPU) in living donors [4]. Many important factors play a role in determining the final intrinsic developmental capacity and quality of the retrieved oocytes. Most of these are donor-related, as extensively reviewed by Merton et al [3]: i.e. stage of the estrous cycle at the time of oocyte retrieval, follicular status in relation to oocyte growth and final maturation, and donor’s physiological condition and breed. In addition, the technical procedures necessary to obtain the immature cumulus oocyte complexes (COCs) have an impact on oocyte quality. Important technical factors are the temperature and time of storage for slaughterhouse ovaries [5], and factors affecting OPU include follicle visualization, needles and aspiration vacuum used, and COC processing before IVP, all of which can jeopardize the quality of the oocyte’s cumulus cell investment [4]. There is general agreement that the importance of an intact cumulus cell investment for oocyte maturation and *in vitro* embryo development cannot be overestimated [6–8]. Moreover, prediction of the developmental potential of the harvested COCs is crucial for an effective oocyte selection. Oocyte quality is most commonly assessed by the ability to further develop to the blastocyst stage *in vitro* [9], which is known to differ in several ways from *in vivo* development. For instance, bovine IVP is generally performed in large groups of COCs, as group culture is still a prerequisite to achieve acceptable blastocyst rates. Unfortunately, group IVP makes studies on *in vitro* oocyte quality linked to the biological data of the donor, or the originating follicle, impossible. To solve this problem, individual IVP might be the solution. But, even when the immediate link between the

individual oocyte and its specific follicular environment is lost or of less interest, the need for *in vitro*, noninvasive oocyte quality assessment techniques remains. Only with such a technique in place will it be possible to select good quality oocytes prior to IVP procedures, to economize the IVP procedure as a whole, and to increase its efficiency that remains limited to about 30–40% blastocysts.

In the first part of this review, noninvasive markers predictive for *in vitro* oocyte developmental competence, irrespective of oocyte origin, are discussed. Due to the scarcity of these predictive procedures, the second part of this review addresses the need for and the possibilities of individual *in vitro* embryo production as a tool to link individual morphological oocyte quality to embryo development, as well as a means to investigate the accuracy of (new) noninvasive oocyte quality assessment parameters. In addition, to the best of our knowledge, this review is the first to discuss procedures available for individual IVP.

2. The need for routine, noninvasive oocyte quality parameters

The developmental competence of oocytes is progressively acquired during follicular growth in the ovary [10]. The relationship between the ovarian and follicular environment (follicle size, grade of follicular atresia, biochemical characteristics of the follicular fluid, estrous cycle, etc.), and the *in vitro* developmental competence of the oocyte is evident. Although useful as a noninvasive predictor of oocyte developmental quality [8,11–17], this connection between the oocyte and its originating follicle cannot always be maintained in a commercial IVP environment.

In OPU-IVP settings, follicular fluid is collected together with the oocyte through follicular aspiration. However, during the OPU session, a variable number of follicles are punctured and their contents are pooled on an embryo filter. If follicular fluid is retrieved for content analysis, each follicle should be aspirated separately, which is technically difficult because of the large dead volume of the aspiration equipment. Recently, a

review in human reproduction describes the obstacles to the analysis of follicular fluid to assess oocyte quality [18]. In addition to the discomfort caused by multiple vaginal punctures and the increased risk for bleeding, the needle needs to be flushed with a standardized amount of medium in order to calculate follicular fluid dilution. In addition, multiple biochemical parameters need to be assessed to increase the predictive power of the analysis. This metabolomic approach is, however, still in a beginning stage [18]. When using a large batch of slaughterhouse derived ovaries, where all collected cumulus oocyte complexes are mixed following aspiration, knowledge about the former follicular environment is lost. These immature COC's need to be sorted according to "quality" before maturation. The aim is to eliminate oocytes with an impaired developmental potential from the production system from the very beginning. This should benefit the developmental capacity of the remaining oocytes and increase the efficacy and efficiency of the IVP procedure.

Ideally, oocyte quality assessment protocols or techniques need to accomplish a number of specific objectives: simple and rapid to perform, inexpensive, allow a high throughput of COCs, have an acceptable reliability, and above all, be noninvasive. It is clear that, at the present time, no oocyte quality assessment method available successfully meets all these demands. The following discusses the possibilities, advantages, and disadvantages of currently available options for noninvasive oocyte quality assessment, irrespective of the COC's origin.

2.1. Morphology

Light microscopic evaluation of COC morphology is the most commonly used method to sort immature COCs by quality. Previously, it has been shown that selection on the basis of both cumulus investment morphology, and the homogeneity of the cytoplasm can improve blastocyst rates [19]. The morphology of the cytoplasm is indeed related to the COC's developmental competence [8], and light microscopic evaluation of its homogeneity is therefore a very easy and rapid tool to assess oocyte quality. Unfortunately, bovine oocytes have a dark ooplasm that can impair assessment; this is in contrast to translucent human oocytes, where granularity, vacuoles, and incorporations are easily identified [20]. Moreover, in bovine COCs, visualization of the extruded polar body and the oocyte's shape and diameter is hampered by a mass of dark cumulus cells. Although several reports stress the importance of oocyte diameter in relation to developmental competence,

most researchers denude bovine oocytes from their cumulus cells in order to measure the oocyte diameter [1,13,21]. That makes this technique invasive and impairs subsequent COC developmental competence. Given the important role of the cumulus cells [22], immature bovine oocytes have long since been divided into different quality categories, mainly based upon evaluation of the compactness of the cumulus cell layers under light microscopy [23,24]. After all, it has been sufficiently documented that IVP using oocytes with an incomplete or damaged cumulus results in a significantly lower blastocyst outcome, compared to culture of COCs with a complete, dense cumulus; the latter achieving the highest cleavage and embryo production rates [3,25]. In contrast, other reports state that COCs with certain characteristics associated with early atresia, beginning expansion in the outer cumulus cell layers, and clumping cytoplasm, show equal or even higher developmental potential than COCs with a complete, compact, multilayered cumulus investment and homogeneous cytoplasm [19,24,26]. The greatest advantage in selecting oocytes for IVP seems to be rejecting COCs with minimal cumulus [19], but the accuracy of this as a predictor of oocyte quality is still in discussion [16]. Indeed, even with the best quality COCs, on the basis of their morphology, we can only achieve approximately 30–40% of blastocysts *in vitro*. This fact strongly suggests that there is a need for additional noninvasive oocyte quality parameters.

2.2. Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PDH) is an enzyme in the pentose phosphate pathway that plays a role in energy supply to the cells by maintaining the level of NADPH. It is particularly active in developing oocytes with a clear decrease in activity when the oocytes have finished their growth phase [27]. Brilliant cresyl blue (BCB), a vital blue dye, is reduced to a colorless substance through the action of G6PDH, and can therefore be a measure for the intracellular activity of G6PDH [28]. Fully grown oocytes show a decreased G6PDH activity, meaning that their cytoplasm will remain blue, following the uptake of BCB (BCB+), while growing oocytes show a colorless cytoplasm (BCB-) after exposure to BCB. Rodríguez-González et al [29], in the prepubertal goat, and Pujol et al [30] in heifers, showed that BCB+ oocytes have a larger diameter and thus are assumed to be more competent. Following maturation, more BCB+ oocytes reach the MII stage compared to BCB- and non-treated oocytes [28,29]. Ishizika et al [31] showed that matured porcine

Table 1

Blastocyst rates (on total number of oocytes) obtained by different research groups after oocyte selection by Brilliant Cresyl Blue (BCB), compared to control oocytes.

Authors	Species (Specific Topic)	Control (%)	BCB+ (%)	BCB– (%)
Pujol et al, 2004	Heifers	5.2	12.3	1.6
Alm et al, 2005	Bovine	19.2	34.1	3.9
Bhojwani et al, 2007	Bovine (SCNT*)	21	39	4
Katska-Książkiewicz et al, 2007	Goat	19.1	13.3	0
Opiela et al, 2008	Bovine	13.2	14.2	7.6
Vandaele et al, 2008	Bovine	24.2	19.2	7.8

* Somatic Cell Nuclear Transfer.

BCB+ oocytes have higher meiotic competences (MII spindle) and intraoocyte glutathione levels, indicating a better cytoplasmatic maturation. Various studies counted the percentage of BCB+ oocytes after oocyte selection solely based on morphology. It differed from 50%, in prepubertal goat oocytes [29], to 58% in bovine oocytes retrieved after slicing the ovaries [28], reaching 75.6% and 65.2% in pig [31,32] and 79% with heifer oocytes [30]. In other words, a variable aspect of the oocytes passed morphological selection, but they should be excluded from IVP on the basis of the BCB test. Additional reports reiterate the value of the BCB test for the selection of developmentally competent oocytes [33] related to bovine nuclear transfer procedures [34], and for use with COCs collected by OPU [35]. Torner et al [36] tried to identify intrinsic molecular and subcellular differences between BCB+ and BCB– oocytes. They used various techniques, such as Western blot analysis for the phosphorylation pattern of protein kinases, a cDNA microarray for gene expression profiles, and fluorescence labelling and photometric measurement for chromatin configuration of the nucleus and the mitochondrial activity of the oocytes. This broad approach undeniably indicated molecular and subcellular organisational variations in oocytes with different G6PDH activity, as demonstrated by dissimilar gene expression patterns and lower mitochondrial activity in BCB+ oocytes. Other studies investigated the correlation between G6PDH activity, assessed via BCB, and the expression of apoptotic genes (Bcl-2 and Bax) or the apoptotic cell ratio [37,38], but no correlation was found.

Most of the cited studies suggest that brilliant cresyl blue might be a good noninvasive oocyte quality indicator to be used in IVP. Indeed, a majority of the reports show a better developmental capacity, resulting in higher blastocyst rates, for BCB+ oocytes, compared to BCB– oocytes. However, no reproducible differences in blastocyst rates can be found between morphologically selected control COCs and BCB+ se-

lected oocytes (Table 1, [37,38]). Moreover, not one study reports a blastocyst rate obtained with BCB selected oocytes higher than the 30 to 40% obtained following routine morphological oocyte selection. Our experiments (unpublished results) revealed that the difference between BCB– and BCB+ oocytes is often subjective. Also, the procedure itself, requiring a 90 minute incubation, makes this technique time consuming, and this can affect the developmental competence of the screened oocytes [38].

2.3. Cumulus cell investigation

As stated earlier, an enormous amount of knowledge has been generated by the study of the interplay between the follicle as a whole, the cumulus cells in particular, and the enclosed oocyte [13,22]. The oocyte plays a key role in regulating the functions of the surrounding cumulus cells [39]. Thus, instead of concentrating on the oocyte itself, assessment of cumulus cell viability and metabolism might tell us something about the developmental potential of the enclosed oocyte. Several groups, mainly in human reproductive research, investigated molecular markers in cumulus cells by gene expression as an approach to gain insight into the genes involved in follicle growth and oocyte developmental quality [40]. Recently, two reports identified potential genes in bovine cumulus cells as markers of oocyte quality through quantitative RT-PCR: hyaluronan synthetase 2 (*HAS2*), inhibin β A (*INHBA*), epidermal growth factor receptor (*EGFR*), gremlin 1 (*GREM1*), betacellulin (*BTC*), CD44, tumor necrosis factor-induced protein 6 (*TNFAIP6*), prostaglandin-endoperoxide synthetase 2 (*PTGS2*) [41], and cathepsins B, S, and Z, where a higher expression of cumulus cell cathepsins would indicate an increase in the number of apoptotic nuclei in the cumulus layer [42]. Although some correlations look very promising, it is still uncertain if cumulus cell characteristics are good predictors of the oocyte's developmental competence. For exam-

ple, the number of apoptotic cumulus cells does not reflect the oocyte competence [16,43]. As yet, expression of cumulus genes as markers for noninvasive assessment of oocyte quality has not been established in a commercial way, not even in humans [44,45]. The evaluation of biochemical and cytological (invasive) parameters require a biopsy of the cumulus cells. While this procedure might not necessarily impair the COC's developmental competence, it is very laborious, complicated, expensive and time-consuming, and therefore, not currently applicable in human clinical practice [18], and thus, even less appropriate for routine application in cattle.

2.4. Future perspectives

More attention is paid to oocyte and embryo metabolism, i.e. net depletion or release of nutrients, such as pyruvate, glucose, lactate and amino acids, and oxygen consumption. Analysis of the medium wherein an oocyte or embryo was cultured can provide information about the corresponding oocyte/embryo quality. Especially regarding embryo metabolism, a number of insights have been recently gained. The "Quiet Embryo Hypothesis" [46] states that the more viable embryos show lower metabolic rates, compared to their less viable counterparts. For example, amino acid turnover has potential as a predictor of embryo quality [47]. In this way, analysis of maturation medium can be a tool to assess oocyte quality [48,49]. However, applying this technique dictates that oocyte selection can only be done following maturation. Moreover, each oocyte needs to be matured individually and their medium has to be analyzed separately. These three requirements make using individual oocyte metabolism as a selection tool for *in vitro* developmental competence unlikely to be implemented routinely in the near future. In the second part of this review, the obstacles and possibilities of individual maturation of bovine oocytes, as well as individual embryo culture, are discussed.

To summarize thus far, the only noninvasive effective quality and selection parameter remaining is COC morphology immediately following follicle aspiration. Although the excellent work of many researchers worldwide [49], using invasive technology to unravel factors determining oocyte quality, has shown that a great number of quality parameters are not morphologically assessable (i.e. mRNA transcription, protein translation, oocyte metabolism, and mitochondria distribution). As the developmental potential of an oocyte is determined by multiple factors, there is an urgent need for additional noninvasive quality assessment pro-

cedures. Establishment of a system that allows the growth of bovine oocytes individually to the blastocyst stage requires that the factors necessary for single development be developed. On the other hand, the ability to grow single embryos will generate new insight into intrinsic oocyte quality, and thus may lead to the identification of promising (new) oocyte quality parameters. The next part of this review describes the state of the art of single bovine oocyte culture and the value of such a system in the search for noninvasive oocyte quality assessment methods.

3. The development of a routine single oocyte culture system

Sirard and coauthors [9] described oocyte competence as the ability to subsequently: 1) resume meiosis; 2) cleave following fertilization; 3) develop to the blastocyst stage; 4) induce pregnancy; and 5) bring offspring to term in good health. As the fourth and fifth step are often difficult and expensive to assess under experimental conditions, at the present time the ability to develop to the blastocyst stage *in vitro* is the key consideration for most laboratories. The accuracy of this endpoint is substantiated by the fact that the normalcy of the oocyte is the main condition necessary for blastocyst formation [9]. As explained above, a comprehensive noninvasive screening method to assess the developmental quality of a retrieved oocyte is still lacking. Testing the accuracy of quality assessment techniques requires a system in which each individual oocyte can be followed until the blastocyst stage. Therefore, ideally, the individual oocyte should survive quality assessment so that it can be re-introduced into the IVP system to subsequently develop to the blastocyst stage and thereby confirm the outcome of the initial quality assessment. Looking specifically at bovine routine *in vitro* embryo production systems, group culture is still a prerequisite to achieve acceptable blastocyst rates. As a consequence, it is impossible to follow an individual oocyte through the IVP procedure and draw conclusions on the developmental capacity of the initial single cumulus oocyte complex. It was also shown that the presence of morphological quality grade I oocytes can facilitate the development of COCs with lower developmental potential [50]. While testing (new) oocyte quality assessment methods in a group culture environment, the positive or negative effects from neighboring developing, and/or degenerating, oocytes can bias conclusions on the developmental competence of individual oocytes. The following para-

graphs discuss the purposes of the use of a single bovine oocyte culture system, the efforts which have already been made to achieve it and the problems that still need to be solved.

3.1. Purposes

Apart from testing the efficacy of noninvasive oocyte quality assessment techniques, the availability of a routine single oocyte culture system can serve several other objectives. Firstly, embryos can be traced back to the follicle from which they originate [51]. From a scientific point of view, tracking individual oocytes with a specific known physiological background will greatly contribute to our knowledge on folliculogenesis and oogenesis. Secondly, intense interest in oocyte and embryo metabolism, characterized by variations in gene-expression patterns and different amounts of transcription and translation products, increases the need for individual production of embryos [52–54]. Thirdly, when *in vitro* embryo production is used for screening purposes, for instance in toxicology studies, single oocyte culture could reduce the size of the sample, as each single embryo can be considered an independent statistical experimental unit, and no group effects have to be taken into account. In addition, separate culture conditions are also preferable to avoid conglomeration of oocytes from which the *zona pellucida* was removed [55], as well as to culture oocytes and embryos resulting from labor-intensive manipulative methods, such as nuclear transfer and genotyping, where individual identification of oocytes is highly preferred [56–57]. In the future, 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 [4,58], and these donors are often of high genetic value or members of endangered species [52,59]. Last but not least, individual bovine *in vitro* embryo production can be a promising model for studying certain aspects of human infertility [60], particularly because in human ART oocytes and embryos are routinely cultured singly.

3.2. Obstacles

In most domestic species, embryo development in the female tract occurs individually, or at least separated by a significant distance. Apparently, 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 have failed, or at least yielded

disappointing results [15,58,59,61–69]. Not only developmental competence as such, but also embryo quality was affected, as shown by embryos with a lower cell number [57], a relatively smaller inner cell mass [70], low hatching rates [52,66,67,71], and low cryotolerance [57]. Only a few groups achieved similar, or even higher blastocyst rates, following single IVP, compared to group culture [16,24,52,55], although embryo quality was not always satisfactory. Our research showed that technical experience in handling individual oocytes and zygotes influences embryo development, as blastocyst rates increased from 21.8 to 31.9% with routine practice and expertise with individual culture, without substantial changes to the protocol [72].

Successful development of embryos in group culture can largely be attributed to autocrine and paracrine communication between oocytes and embryos. These 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) [61,73–75]. The concentration and dilution of several embryo-derived stimulatory factors seems to be crucial. Gopichandran et al [76] found the distance of 165 μm between zygotes 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 influences the development in a negative way [77]. Single oocyte culture seems to be compromised by the lack of interaction with other oocytes/embryos. In order 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 following paragraphs consider a number of possibilities to ameliorate single development, including: medium supplementation, somatic cell co-culture, gas atmosphere composition, medium replacement, droplet size, and a modified physical environment.

3.3. Medium supplementation

To counteract the low levels of growth factors, standard culture media are supplemented with a variety of additional growth factors and 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 summary, one of the most controversial topics is the use of serum. According to Carolan et

al [52], the use of serum during individual maturation is detrimental, while others did not report this negative effect [53]. When singly cultured in serum-free medium, the addition of amino acids and vitamins had a positive effect on the development of flushed sheep zygotes [78]. Li et al [79] could enhance single bovine embryo development by adding amino acids to a chemically defined culture medium, at concentrations found in the female reproductive tract. Furthermore, the effect of the addition of several other defined supplements was tested. Mizushima and Fukui [56] added hypotaurine and β -mercaptoethanol to the maturation medium of single oocytes that resulted in significantly higher rates of normal fertilization and lower rates of polyspermy, compared to the supplementation of hypotaurine alone. Adding either β -mercaptoethanol or both however, showed no significant differences in cleavage rates, but tended to improve blastocyst development compared to culture without additions (6.4 and 6.8% versus 1.6%). The presence of a high glucose concentration seemed to be beneficial for IVM under low oxygen tension conditions, while the addition of EGF and/or cysteamine to the maturation medium improved fertilizability, developmental competence, and cryoresistance [80]. This group also showed that the addition of EGF to a group-IVP system did not affect the blastocyst rate, whereas EGF addition clearly increased the number of blastocysts in the individual-IVP system [80]. The addition of glutathione during single oocyte IVF significantly increased the proportion of normal fertilization and decreased polyspermy [54]. Lim and Hansel [74] demonstrated the positive effects of the addition of platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF $\beta_1\beta_2$) on the developmental capacity of singly cultured 8-cell embryos. Arachidonic acid, β -mercaptoethanol and glutathione stimulated the subsequent development of 8-cell embryos in the presence of PDGF and TGF $\beta_1\beta_2$.

3.4. Co-culture

As an alternative to medium supplementation, culture environment can be enriched by culturing somatic cells together with the zygote. In this way, individual oocyte culture was performed with co-culture of bovine oviductal epithelial cells (BOEC) [5], Buffalo rat liver cells (BRL) [63], cumulus cells [63,72], or granulosa cells [59]. This renews the debate whether or not co-culture systems with somatic cells should be used in IVP systems. Despite the fact that the use of co-culture undoubtedly increases the amount of non-defined fac-

tors in the production system, the added cells can support embryo development and improve embryo quality by secreting embryotrophic factors and neutralizing embryo toxic components [81,82]. Recently, our group tested the effect of cumulus cell (CC) co-culture on single embryo culture [72]. Cumulus oocytes complexes from slaughterhouse ovaries were routinely matured and fertilized in groups and then assigned to 4 different culture treatments: group or individual culture, with or without the addition of CC. Co-culture with CC was significantly ($P < 0.001$) beneficial to blastocyst rates in single culture conditions (21.8% with CC versus 2.9% without CC), but not in group culture conditions (24.6% with CC and 30.7% without CC) [72]. In a subsequent experiment, two different single culture systems were tested: individual culture on a cumulus cell monolayer grown for 5 days before the start of culture, or adding cumulus cells recycled from the fertilization medium at the start of culture. Both culture systems fully supported individual culture, as no significant difference in blastocyst rates with group cultured zygotes could be found (38.2 and 31.9% respectively, compared to group culture: 36.4%). Moreover, mean cell numbers of the embryos were not significantly different, indicating comparable embryo quality between singly and group cultured blastocysts. An alternative way, though equally undefined, to achieve paracrine support and stimulation of singly cultured oocytes, is using conditioned media, in which cumulus-oocyte complexes or somatic cells were previously incubated. In the latter experiment, we also tested cumulus cell conditioned medium, but only 6.4% of the zygotes developed to the blastocyst stage [72]. This is in agreement with Hagemann et al [53], while the use of conditioned media derived from embryo culture in groups increased the blastocyst formation of individual cultured embryos for Fujita et al [68].

3.5. Gas atmosphere

Gas atmosphere during *in vitro* development can also change the biological microenvironment, and thus influence embryo metabolism [49] due to the potential link with the production of reactive oxygen species (ROS). Hagemann et al [53] did not find a significant difference in developmental rates when using 7% or 20% O_2 during maturation or fertilization of single bovine oocytes, while according to Oyamada et al [80] cleavage and blastocyst rates were significantly higher 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 [83]. In the presence

of a cumulus cell co-culture in SOF medium, 5% oxygen increased blastocyst rates and embryo cell numbers significantly, compared to 20% oxygen, in both group and single culture [72], although in general, 20% oxygen is preferred in case a somatic cell co-culture is used [84].

3.6. Medium replacement and droplet size

To create an equilibrium between the dilution of autocrine factors, the accumulation of toxic metabolites and the availability of nutrients during 7 days of culture, a medium replacement schedule can be applied [62], but as far as we know, it was never tested for individual culture. Another way to maintain this equilibrium is to change the droplet size, as substrate depletion and build-up of toxic substances can occur in micro-drops. In this way, single embryo culture was shown to be compromised in droplet sizes smaller than 10 μL [52,59,64], however, single fertilization in 10 μL was shown to be better than in 25, 50 or 100 μL [54].

3.7. Physical environment and dynamic culture conditions

To mimic the *in vivo* situation, where the embryo is surrounded by small amounts of reproductive tract secretions wherein growth factors are available at high concentrations, but keep embryos individually identifiable at the same time, Vajta et al [55] constructed the “well of the well” system (WOW) for *in vitro* culture. At the bottom of a standard 4-well plate, 10 to 15 V-shaped smaller wells were melted with a polished steel rod. The 4 large wells were filled with 500 μL of culture medium and covered with oil. In each V-shaped well a zygote, with or without the *zona pellucida*, was cultured. While the volume of each V-shaped well was only 0.04 μL , limiting the dilution of autocrine factors, the large amount of medium above the small wells supposes to provide nutrients and dilute toxic metabolites. This culture system resulted in higher blastocyst rates than conventional group culture or single culture in droplets of 20 μL , and the blastocyst cell number equalled that of group cultured embryos [55]. This system allows identification of single oocytes and zygotes, but the zygotes are covered by the same medium (Figure 1a) allowing inter-embryonic communication to influence neighbouring developing zygotes that can be undesirable if one is interested in individual developmental characteristics of a specific embryo. Hoelker et al [77] showed comparable blastocyst rates, differential cell counts, and apoptotic indexes in WOW culture with 16 zygotes in shared medium, compared to

Fig. 1. Two types of the “well of the well” (WOW) system: (a) one zygote per microwell and all microwells are covered by a large volume of medium; (b) each microwell with 1 zygote is covered by an individual droplet of medium.

control group culture with 50 zygotes per droplet, while a culture of 16 zygotes in a standard droplet decreased these outcomes. In human, pregnancy was efficiently established after using the WOW system [85]. Another application of this “apart together” culture, to allow both group stimulation and individual identification, is to culture one zygote separated from a group of embryos by a mesh barrier [86,87]. Han et al (2006) [16] developed a WOW comparable system, a well-in-drop (WID) culture system, for single goat oocytes, while others [57,88] modified the WOW system slightly by covering each individual small well with 20 μL medium (Fig. 1b) and using co-culture with cumulus cells in a high O_2 atmosphere. Under these conditions, no common medium over-layer is used, but lipophilic factors can be absorbed in the oil and diffuse into other medium droplets. They did not report higher blastocyst rates, compared to an individual 20 μL standard droplet culture system, but cell numbers of modified WOW cultured embryos were higher. Our own experience (Fig. 2) revealed that moulding the wells in the bottom of culture plates is labor-intensive and time-consuming. Moreover, culture plates containing zygotes in the mini-wells need to be transported with extreme care to avoid embryos becoming dislodged by an abrupt movement. In addition, individual culture in 20 μL droplets in individual wells was not enhanced by culture in microwells in the presence of a cumulus cell co-culture (Table 2 [89]). In agreement, another study [90] compared three “apart together” systems: WOW, adhesive Cell-Tak, and a polyester mesh, resulting in comparable developmental outcomes but with a preference for the latter in terms of easier preparation and use.

A few novel physical environments for embryo culture, such as microfluidic devices [91,92] and glass oviducts or microtube culture [93,94] were developed.

Fig. 2. (a) A morula in a modified “well of the well”, in a droplet of 20 μ l medium under oil. (b) A hatched blastocyst which exceeds the miniwell volume.

In contrast to microdrops under oil, very small volumes of medium can be used without oil overlay. The microfluidic devices allow a dynamic environment where the fluid flow can wash toxic metabolites away, but it simultaneously dilutes autocrine/paracrine factors. These huge differences in physical environment are likely to affect individual embryo development very differently, and might prove promising for individual production of bovine embryos, but have not yet been tested under routine conditions.

It is clear that individual culture of (bovine) oocytes is compromised compared to group culture. To overcome low blastocyst rates and impaired embryo quality many modifications to the routine *in vitro* production protocols were tested, with variable success. The method of choice depends on the indication for the use of a single oocyte culture system. A chemically defined medium without co-culture, is most likely essential to study oocyte and embryo metabolism. If single oocyte culture systems are used in a commercial setting, where the aim is to produce the highest possible number of good quality embryos from a limited number of oocytes, the use of serum and co-culture systems can be

beneficial. The more various methods that are tested to obtain an efficient single oocyte culture system, the more knowledge on the specific needs of a single oocyte or embryo will be generated. Individual embryo production can help to unravel the complex interactions between the oocyte and somatic cells without the influence of other oocytes. A few research groups used single oocyte culture as a tool, for example to link follicle environment and cumulus-oocyte complex characteristics to the developmental competence of the oocyte involved [14–16,95].

4. Conclusions

Despite multiple attempts to improve blastocyst rates by modifying *in vitro* culture conditions for bovine embryos, mean blastocyst rates have not routinely been lifted above 30–40%. Selection of the oocytes with the highest developmental competence, acquired during follicular growth, seems to be the key factor for IVP success. Because this quality assessment needs to be noninvasive and routinely applicable on a large scale, only a few assessment methods are currently available. Light microscopic grading of the morphological quality of the cumulus oocyte complexes is the method of choice in the majority of laboratories, as this technique has proven its efficacy. To increase the blastocyst rates, other complementary noninvasive methods need to be established in order to identify and track ultrastructural aberrations.

Interestingly, research on the effectiveness of such quality assessment protocols requires a system where each evaluated oocyte can be followed until blastocyst stage. Unfortunately, single *in vitro* embryo production is not yet a standard technique, as individual development is hampered compared to group culture. Nevertheless, several efforts have been made which might lead to better individual developmental outcomes. Only by combining research on additional noninvasive oocyte quality assessment techniques with efforts on the development of single oocyte culture protocols to test

Table 2

Cleavage, blastocyst, and hatching rates of bovine zygotes cultured in groups vs. individual on a cumulus cell monolayer or on a monolayer combined with a modified well of the well (mWOW).

Treatment	Nr of oocytes	Cleavage (%)*	Blastocysts D8 (%)*	% Hatched D10*
Group control	207	141 (68.1)	58 (28.0)	67.2
Single control	182	137 (75.3)	51 (28.0)	60.7
Single mWOW	168	123 (73.2)	44 (26.2)	65.3

* No significant differences could be found in the same row.

these techniques, can we improve the success of bovine *in vitro* embryo production substantially.

Acknowledgements

The authors thank M. Julian (JustMe Editing, Storrs, CT) for editing and critical reading of the manuscript.

References

- [1] Hasler JF. The current status of oocyte recovery, *in vitro* embryo production, and embryo transfer in domestic animals, with an emphasis on the bovine. *J Anim Sci* 1998;76(3 Suppl):52–74.
- [2] Galli C, Crotti G, Notari C, Turini P, Duchi R, Lazzari G. Embryo production by ovum pick up from live donors. *Theriogenology* 2001;55:1341–57.
- [3] Merton JS, de Roos APW, Mullaart E, de Ruigh L, Kaal L, Vos PLAM, Dieleman SJ. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology* 2003;59:651–74.
- [4] Bols PEJ. Puncture of immature ovarian follicles in bovine assisted reproduction. *Verhandelingen* 2005;LXVII,3:177–02.
- [5] Blondin P, Coenen K, Guilbault LA, Sirard MA. In vitro production of bovine embryos: developmental competence is acquired before maturation. *Theriogenology* 1997;47:1061–75.
- [6] Zhang L, Jiang S, Wozniak PJ, Yang X, Godke RA. Cumulus cell function during bovine oocyte maturation, fertilization and embryo development *in vitro*. *Mol Reprod Dev* 1995;40:338–44.
- [7] Konishi M, Aoyagi Y, Takedomi T, Itakura H, Itoh T, Yazawa S. Presence of granulosa cells during oocyte maturation improved *in vitro* development of IVM-IVF bovine oocytes that were collected by ultrasound-guided transvaginal aspiration. *Theriogenology* 1996;45:573–81.
- [8] Boni R, Cuomo A, Tosti E. Developmental potential in bovine oocytes is related to cumulus-oocyte complex grade, calcium current activity, and calcium stores. *Biol Reprod* 2002;66:836–42.
- [9] Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. *Theriogenology* 2006;65:126–36.
- [10] Mermillod P, Dalbiès-Tran R, Thélie A, Traverso JM, Perreau C, Papillier P, Monget P. Factors affecting oocyte quality: Who is driving the follicle? *Reprod Dom Anim* 2008;43(suppl.2):393–400.
- [11] Lonergan P, Monaghan P, Rizo D, Boland MP, Gordon I. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture *in vitro*. *Mol Reprod Dev* 1994;37:48–53.
- [12] Gandolfi F, Luciano AM, Modina S, Ponzini A, Pocar P, Armstrong DT, Lauria A. The *in vitro* developmental competence of bovine oocytes can be related to the morphology of the ovary. *Theriogenology* 1997;48:1153–60.
- [13] Fair T. Follicular oocyte growth and acquisition of developmental competence. *Anim Reprod Sci* 2003;78:203–16.
- [14] Hagemann LJ, Beaumont SE, Berg M, Donnison MJ, Ledger A, Peterson AJ, Schurmann A, Tervit HR. Development during single IVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. *Mol Reprod Dev* 1999;53:451–8.
- [15] Jewgenow K, Heerdegen B, Müller K. *In vitro* development of individually matured bovine oocytes in relation to follicular wall atresia. *Theriogenology* 1999;51:745–56.
- [16] Han Z, Lan G, Wu Y, Han D, Feng W, Wang J, Tan J. Interactive effects of granulosa cell apoptosis, follicle size, cumulus-oocyte complex morphology, and cumulus expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. *Reproduction* 2006;132:749–58.
- [17] Sinclair KD, Lunn LA, Kwong WY, Wonnacott K, Linforth RST, Craigmiles J. Amino acid and fatty acid composition of follicular fluid as predictors of in-vitro embryo development. *Reprod BioMed Online* 2008;16:859–68.
- [18] Revelli A, Piane LD, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrinol* 2009;7:40–52.
- [19] Hawk HW, Wall RJ. Improved yields of bovine blastocysts from in vitro-produced oocytes. I. Selection of oocytes and zygotes. *Theriogenology* 1994;41:1585–94.
- [20] Borini A, Lagalla C, Cattoli M, Sereni E, Sciajno R, Flamigni C, Coticchio G. Predictive factors for embryo implantation potential. *Reprod BioMed Online* 2005;10:653–68.
- [21] Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Mol Reprod Dev* 1995;42:437–42.
- [22] Tanghe S, van Soom A, Nauwynck H, Coryn M, de Kruif A. Mini-review: Functions of the cumulus oophorus during oocyte maturation, ovulation and fertilization. *Mol Reprod Dev* 2002;61:414–24.
- [23] de Loos F, Van Vliet C, Van Maurik P, Kruip ThAM. Morphology of immature bovine oocytes. *Gamete Research* 1989;24:197–204.
- [24] Hazeleger NL, Hill DJ, Stubbings RB, Walton JS. Relationship of morphology and follicular fluid environment of bovine oocytes to their developmental potential *in vitro*. *Theriogenology* 1995;43:509–22.
- [25] Shioya Y, Kuwayama M, Fukushima M, Iwasaki S, Hanada A. *In vitro* fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cells and matured *in vitro*. *Theriogenology* 1988;30:489–96.
- [26] Blondin P, Sirard MA. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol Reprod Dev* 1995;41:54–62.
- [27] Mangia F, Epstein CJ. Biochemical studies of growing mouse oocytes: preparation of oocytes and analysis of glucose-6-phosphate dehydrogenase and lactate dehydrogenase activities. *Dev Biol* 1975;45:211–20.
- [28] Alm H, Torner H, Löhke B, Viergutz T, Ghoneim IM, Kanitz W. Bovine blastocyst development rate *in vitro* is influenced by selection of oocytes by brilliant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. *Theriogenology* 2005;63:2194–205.
- [29] Rodríguez-González E, López-Béjar M, Velilla E, Paramio MT. Selection of prepubertal goat oocytes using the brilliant cresyl blue test. *Theriogenology* 2002;57:1397–409.
- [30] Pujol M, López-Béjar M, Paramio MT. Developmental competence of heifer oocytes selected using the brilliant cresyl blue (BCB) test. *Theriogenology* 2004;61:735–44.
- [31] Ishizaki C, Watanabe H, Bhuiyan MMU, Fukui Y. Developmental competence of porcine oocytes selected by brilliant

- cresyl blue and matured individually in a chemically defined culture medium. *Theriogenology* 2009;72:72–80.
- [32] Roca J, Martinez E, Vazquez JM, Lucas X. Selection of immature pig oocytes for homologous *in vitro* penetration assays with brilliant cresyl blue test. *Reprod Fertil Dev* 1998;10:479–85.
- [33] Sugulle A, Katakawa S, Yamamoto S, Oomori S, Ito I, Dochi O, Koyama H. Selection of bovine oocytes by brilliant cresyl blue before *in vitro* maturation improves blastocyst development. *Reprod Fertil Dev* 2006;19:273.
- [34] Bhojwani S, Alm H, Torner H, Kanitz W, Poehland R. Selection of developmentally competent oocytes through brilliant cresyl blue stain enhances blastocyst development rate after bovine nuclear transfer. *Theriogenology* 2007;67:341–5.
- [35] Tagawa M, Matoba S, Okada M, Metoki K, Imai K. Developmental competence of oocytes selected by the brilliant cresyl blue staining in prepubertal and adult cattle. *Reprod Fertil Dev* 2006;19:273–4.
- [36] Torner H, Ghanem N, Ambros C, Hölker M, Tomek W, Phatsara C, Alm H, Sirard MA, Kanitz W, Schellander K, Tesfaye D. Molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. *Reproduction* 2008;135:197–212.
- [37] Opiela J, Katska-Ksiazkiewicz L, Lipinski D., Slomski R, Bzowska M, Rynska B. Interactions among activity of glucose-6-phosphate dehydrogenase in immature oocytes, expression of apoptosis-related genes Bcl-2 and Bax, and developmental competence following IVP in cattle. *Theriogenology* 2008;69:546–55.
- [38] Vandaele L. PhD Thesis, ISBN 978-90-586-4150-2, 2008; Chapter 5.2. p. 109–23.
- [39] Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* 2002;296:2178–80.
- [40] Li Q, McKenzie LJ, Matzuk MM. Revisiting oocyte-somatic cell interactions: in search of novel intrafollicular predictors and regulators of oocyte developmental competence. *Mol Hum Reprod* 2008;14:673–8.
- [41] Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S, Sirard MA. Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate *in vitro*. *Biol Reprod* 2008;79:209–22.
- [42] Bettgowda A, Patel OV, Lee KB, Park KE, Salem M, Yao J, Ireland JJ, Smith GW. Identification of novel bovine cumulus cells molecular markers predictive of oocyte competence: functional and diagnostic implications. *Biol Reprod* 2008;79:301–9.
- [43] Anguita B, Vandaele L, Mateusen B, Maes D, Van Soom A. Developmental competence of bovine oocytes is not related to apoptosis incidence in oocytes, cumulus cells and blastocysts. *Theriogenology* 2007;67:537–49.
- [44] Anderson R, Scioro R, Bayne R, Kinnell H, Thong K, De Sousa P, Pickering S. Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. *Reproduction* 2009;138:629–37.
- [45] Assou S, Haouzi D, De Vos J, Hamamah S. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Mol Hum Reprod* 2010; in press, doi:10.1093/molehr/gaq032.
- [46] Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays* 2002;24:845–49.
- [47] Sturmey RG, Brison DR, Leese HJ. Assessing embryo viability by measurement of amino acid turnover. *Reprod BioMed Online* 2008;17:486–96.
- [48] Spindler RE, Pukazhenthi BS, Wildt DE. Oocyte metabolism predicts the development of cat embryos to blastocyst *in vitro*. *Mol Reprod Dev* 2000;56:163–71.
- [49] Krisher RL. The effect of oocyte quality on development. *J Anim Sci* 2004;82(E. Supl.):E14–E23.
- [50] Khurana NK, Niemann H. Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. *Theriogenology* 2000;54:741–56.
- [51] Oyamada T, Fukui Y. Oxygen tension and medium supplements for *in vitro* maturation of bovine oocytes cultured individually in a chemically defined medium. *J Reprod Dev* 2004;50:107–17.
- [52] Carolan C, Lonergan P, Khatir H, Mermillod P. *In vitro* production of bovine embryos using individual oocytes. *Mol Reprod Dev* 1996;45:145–50.
- [53] Hagemann LJ, Weibert LL, Beaumont SE, Tervit HR. Development of bovine embryos in single *in vitro* production (sIVP) systems. *Mol Reprod Dev* 1998;51:143–7.
- [54] Fukui Y, Kikuchi Y, Kondo H, Mizushima S. Fertilizability and developmental capacity of individually cultured bovine oocytes. *Theriogenology* 2000;53:1553–65.
- [55] Vajta G, Peura TT, Holm P, Paldi A, Greve T, Trounson AO, Callesen H. New method for culture of zona-included or zona-free embryos: the well of the well (WOW) system. *Mol Reprod Dev* 2000;55:256–64.
- [56] Mizushima S, Fukui Y. Fertilizability and developmental capacity of bovine oocytes cultured individually in a chemically defined maturation medium. *Theriogenology* 2001;55:1431–45.
- [57] Pereira DC, Dode MAN, Rumpf R. Evaluation of different culture systems on the *in vitro* production of bovine embryos. *Theriogenology* 2005;63:1131–41.
- [58] Ward FA, Lonergan P, Enright BP, Boland MP. Factors affecting recovery and quality of oocytes for bovine embryo production *in vitro* using ovum pick-up technology. *Theriogenology* 2000;54:433–46.
- [59] O'Doherty EM, Wade MG, Hill JL, Boland MP. Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology* 1997;48:161–9.
- [60] Campbell B, Souza C, Gong J, Webb R, Kendall N, Marsters P, Robinson G, Mitchell A, Telfer EE, Baird DT. Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans. *Reproduction Suppl* 2003;61:429–43.
- [61] Paria BC, Dey SK. Preimplantation embryo development *in vitro*: cooperative interactions among embryos and role of growth factors. *Proceedings of the National Academy of Sciences of the United States of America* 1990;87:4756–60.
- [62] Ferry L, Mermillod P, Massip A, Dessy F. Bovine embryos cultured in serum-poor oviduct-conditioned medium need co-operation to reach the blastocyst stage. *Theriogenology* 1994;42:445–53.
- [63] Donnay I, Van Langendonck A, Auquier P, Grisart B, Vansteenbergue A, Massip A, Dessy F. Effects of co-culture and embryo number on the *in vitro* development of bovine embryos. *Theriogenology* 1997;47:1549–61.
- [64] Nagao Y, Saeki K, Nagai M. Effects of embryo density, oxygen concentration and medium composition on *in vitro* development of bovine embryos. *Theriogenology* 1998;49:210.
- [65] Palasz AT, Thundathil J. The effect of volume of culture medium and embryo density on *in vitro* development of bovine embryos. *Theriogenology* 1998;49:212.

- [66] Hendriksen PJM, Bevers MM, Dieleman SJ. Single IVP using BRL cell co-culture and serum yields a lower blastocyst rate than a group culture. *Theriogenology* 1999;51:319.
- [67] Yuan YQ, Van Soom A, Laevens H, Coopman F, Peelman L, de Kruif A. Single embryo culture affects hatching rate in bovine *in vitro*-produced embryos. *Theriogenology* 2000;53:307.
- [68] Fujita T, Umeki H, Shimura H, Kugumiya K, Shiga K. Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. *J Reprod Dev* 2006;52:137–42.
- [69] Feng WG, Sui HS, Han ZB, Chang ZL, Zhou P, Liu DJ, Bao S, Tan JH. Effects of follicular atresia and size on the developmental competence of bovine oocytes: A study using the well-in-drop culture system. *Theriogenology* 2007;67:1339–50.
- [70] Ahern TJ, Gardner DK. Culturing bovine embryos in groups stimulates blastocyst development and cell allocation to the inner cell mass. *Theriogenology* 1998;49:194.
- [71] Larson MA, Kubisch HM. The effects of group size on development and interferon- τ secretion by in-vitro fertilized and cultured bovine blastocysts. *Hum Reprod* 1999;14:2075–79.
- [72] Goovaerts IGF, Leroy JMLR, Van Soom A, De Clercq JBP, Andries S, Bols PEJ. Effect of cumulus cell coculture and oxygen tension on the *in vitro* developmental competence of bovine zygotes cultured singly. *Theriogenology* 2009;71:729–38.
- [73] Thibodeaux JK, Myers MW, Hansel W. The beneficial effects of incubating bovine embryos in groups are due to platelet-derived growth factor. *Theriogenology* 1995;43:336.
- [74] Lim JM, Hansel W. Roles of growth factors in the development of bovine embryos fertilized *in vitro* and cultured singly in a defined medium. *Reprod Fertil Dev* 1996;8:1199–205.
- [75] O'Neill C. Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos *in vitro*. *Biol Reprod* 1997;56:229–37.
- [76] Gopichandran N, Leese HJ. The effect of paracrine/autocrine interactions on the *in vitro* culture of bovine preimplantation embryos. *Reproduction* 2006;131:269–77.
- [77] Hoelker M, Rings F, Lund Q, Ghanem N, Phatsara C, Griesse J, Schellander K, Tesfaye D. Effect of microenvironment and embryo density on developmental characteristics and gene expression profile of bovine preimplantative embryos cultured *in vitro*. *Reproduction* 2009;137:415–25.
- [78] Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod* 1994;50:390–400.
- [79] Li R, Wen L, Wang S, Bou S. Development, freezability and amino acid consumption of bovine embryos cultured in synthetic oviductal fluid (SOF) medium containing amino acids at oviductal or uterine-fluid concentrations. *Theriogenology* 2006;66:404–14.
- [80] Oyamada T, Iwayama H, Fukui Y. Additional effect of epidermal growth factor during *in vitro* maturation for individual bovine oocytes using a chemically defined medium. *Zygote* 2004;12:143–50.
- [81] de Wit AAC, Kruip ThAM. Bovine cumulus-oocyte-complex-quality is reflected in sensitivity for α -amanitin, oocyte-diameter and developmental capacity. *Anim Reprod Sci* 2001;65:51–65.
- [82] Orsi NM, Reischl JB. Mammalian embryo co-culture: Trials and tribulations of a misunderstood method. *Theriogenology* 2007;67:441–58.
- [83] Berthelot F, Terqui M. Effects of oxygen, CO₂/pH and medium on the *in vitro* development of individually cultured porcine one- and two-cell embryos. *Reprod Nutr Dev* 1996;36:241–51.
- [84] Yuan YQ, Van Soom A, Coopman FOJ, Mintiens K, Boerjan ML, Van Zeveren A, de Kruif A, Peelman LJ. Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured *in vitro*. *Theriogenology* 2003;59:1585–96.
- [85] Vajta G, Korosi T, Du Y, Nakata K, Ieda S, Kuwayama M, Nagi ZP. The Well-of-the-Well system: an efficient approach to improve embryo development. *Reprod Biomed Onl* 2008;17:73–81.
- [86] Spindler RE, Crichton EG, Agca Y, Loskutoff N, Critser J, Gardner DK, Wildt DE. Improved felid embryo development by group culture is maintained with heterospecific companions. *Theriogenology* 2006;66:82–92.
- [87] Somfai T, Inaba Y, Aikawa Y, Ohtake M, Kobayashi S, Akai T, Hattori H, Konoshi K, Imai K. *Reprod Dom Anim* 2009 [in press].
- [88] Dode MAN, Pereira DC, Rumpf R. Evaluation of different methods for *in vitro* culture of a single bovine embryo. *Theriogenology* 2003;59:340.
- [89] Goovaerts IGF, Leroy JMLR, De Clercq JBP, Andries A, Bols PEJ. Single culture in well of the well does not improve the *in vitro* developmental capacity of bovine embryos cultured on a cumulus cell monolayer. *Proceedings of the 24th Scientific Meeting of the European Embryo Transfer Association (AETE)*, Pau, France, 12th–13th September 2008. p. 160.
- [90] Matoba S, Fair T, Lonergan P. Maturation, fertilisation and culture of bovine oocytes and embryos in an individually identifiable manner: a tool for studying oocyte developmental competence. *Reprod Fertil Steril* 2010;22:839–51.
- [91] Smith GD, Takayama S. Gamete and embryo isolation and culture with microfluidics. *Theriogenology* 2007;68S:S190–S195.
- [92] Wheeler MB, Walters EM, Beebe DJ. Toward culture of single gametes: The development of microfluidic platforms for assisted reproduction. *Theriogenology* 2007;68S:S179–89.
- [93] Thouas GA, Jones GM, Trounson AO. The 'GO' system – a novel method of microculture for *in vitro* development of mouse zygotes to the blastocyst stage. *Reproduction* 2003;123:161–9.
- [94] Roh S, Choi YJ, Min BM. A novel microtube culture system that enhances the *in vitro* development of parthenogenetic murine embryos. *Theriogenology* 2008;69:262–7.
- [95] Araki N, Sato K, Hayashi K, Miyamoto A, Fukui Y. Relationships among follicular fluid estradiol-17 β concentration, morphology of cumulus-oocyte complex and developmental capacity of individual matured, fertilized and cultured bovine embryos *in vitro*. *J Reprod Dev* 1998;44:359–65.