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Review

Oocyte selection for *in vitro* embryo production in livestock species: non-invasive approaches for the new challenges of oocyte competence

Luis Aguila[1]*, Favian Treulen[2], Jacinthe Therrien[3], Ricardo Felmer[4], Martha Valdivia[5] and Smith Lawrence C[6]

[1] Centre de Recherche en Reproduction et Fértilité (CRRF), Université de Montréal, St-Hyacinthe, QC, Canada; luis.aguila.paredes@gmail.com
[2] School of Medical Technology, Faculty of Science, Universidad Mayor, Temuco, Chile; faviantreulen@gmail.com
[3] Centre de Recherche en Reproduction et Fértilité (CRRF), Université de Montréal, St-Hyacinthe, QC, Canada; jacinthe.therrien@umontreal.ca
[4] Laboratory of Reproduction, Centre of Reproductive Biotechnology (CEBIOR-BIOREN), Faculty of Medicine, Universidad de La Frontera, Temuco, Chile; ricardo.felmer@ufrontera.cl.
[5] Laboratory of Animal Reproductive Physiology, Biological Sciences Faculty, Universidad Nacional Mayor de San Marcos, Lima, Peru; mvaldiviac@unmsm.edu.pe
[6] Centre de Recherche en Reproduction et Fértilité (CRRF), Université de Montréal, St-Hyacinthe, QC, Canada; lawrence.c.smith@umontreal.ca
* Correspondence: luis.aguila.paredes@gmail.com

Simple Summary: The efficiency of producing embryos using in vitro technologies in livestock species remains lower when compared to mice, indicating that the proportion of female gametes that fail to develop after *in vitro* manipulation is considerably large. Considering that the intrinsic quality of the oocyte is one of the main factors affecting embryo production, the precise identification of non-invasive markers that predict oocyte competence is of major interest. The aim of this review was to explore the current literature on different non-invasive markers associated with oocyte quality in mammalian species, with an emphasis on the bovine model. Apart from some controversial findings, the presence of cycle-related structures in ovaries, a follicle size between 6 and 10 mm, a large slightly expanded investment without dark areas, large oocyte diameter (>120 microns), dark cytoplasm, and the presence of a round and smooth first polar body have been associated to better embryonic development. In addition, the combination of oocyte and zygote selection and spindle imaging have the potential to further optimize the identification of oocytes with better developmental competence for *in vitro* technologies in livestock species.

Abstract: The efficiency of producing embryos using in vitro technologies in livestock species rarely exceeds the 30 to 40% threshold, indicating that the proportion of oocytes that fail to develop after *in vitro* fertilization and culture is considerably large. Considering that the intrinsic quality of the oocyte is one of the main factors affecting blastocyst yield, the precise identification of non-invasive cellular or molecular markers that predict oocyte competence is of major interest to research and practical applications. The aim of this review was to explore the current literature on different non-invasive markers associated with oocyte quality in mammalian species, with an emphasis on the bovine model. Apart from some controversial findings, the presence of cycle-related structures in ovaries, a follicle size between 6 and 10 mm, large number of surrounding cumulus cells, slightly expanded investment without dark areas, large oocyte diameter (>120 microns), dark cytoplasm, and the presence of a round and smooth first polar body have been associated to better competence. In addition, the combination of oocyte and zygote selection by BCB test and spindle imaging have the potential to further optimize the identification of oocytes with better developmental competence for *in vitro*-derived technologies in livestock species.

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Keywords: oocyte competence; livestock production; assisted reproductive technology; embryo development; micromanipulation; in vitro production

1. Introduction

In recent years, new knowledge in the field of assisted reproductive technologies (ART), has allowed researchers and practitioners to reach new hallmarks in oocyte and sperm *in vitro* competence. Gamete competence is the ability to undergo successful fertilization and develop a normal blastocyst that is capable of implanting in the uterus and generate viable offspring [Reviewed by 1]. Many researchers are working to identify cellular and molecular markers to select the most competent oocyte and spermatozoon to produce embryos with higher implantation potential [Reviewed by 2].

Although it is well known that the most common applications of ARTs in livestock species are for research purposes, some techniques, particularly *in vitro* embryo production (IVP), have become commercially viable and are extensively used for animal breeding [3]. Nonetheless, the efficiency of IVP technologies in livestock species, such as bovine, equine and porcine, measured as the proportion of immature oocytes that reach the blastocyst stage, rarely exceeds the 30 to 40% threshold [4], which means that the proportion of oocytes that fail to develop following *in vitro* maturation, fertilization and culture is considerably large. Contrary to humans, where eggs are mainly collected from preovulatory follicles, in livestock species the oocytes have to be matured *in vitro* due to the difficulty of obtaining a sufficient amount of *in vivo* matured oocytes [5]. Additionally, given that the most frequent source of ovaries are slaughterhouse-derived animals, many important factors that influence oocyte quality, such as age of the donor, the stage of the estrous cycle, nutritional status, genetic potential, presence of a reproductive disorder, and others, are often unknown [Reviewed by 6]. Therefore, it is almost impossible to avoid the retrieval of a heterogeneous population of oocytes that have a distinct ability to undergo maturation and support early embryonic development after fertilization, which is known as developmental competence or oocyte quality [7].

Considering that the intrinsic quality of the oocyte is the main factor affecting blastocyst yield [8], and that embryo culture conditions have a crucial role in determining blastocyst quality [9], the precise selection of mature oocytes that are healthy and have a high developmental competence is vital for IVP technologies in livestock. Usually, for IVP and micromanipulation procedures, such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) and cloning (SCNT), oocyte selection is based on non-invasive features that are easily assessed with light microscopy [Reviewed by 10]. The major difference and/or advantage of conventional IVF compared to micromanipulation procedures is that fertilization can occur during gamete co-incubation when the oocyte has reached, or is close to nuclear and cytoplasmic maturity [11]. Conversely, during micromanipulation procedures, the operator must accurately assess the maturity of the oocyte, and, therefore, its competence [12]. Because the criteria used for grading and selecting oocytes vary among researchers, could be easily misinterpreted and depends on the expert's evaluation and experience, the identification of non-invasive cellular or molecular markers that predict oocyte competence is a major research goal [13,14]. Despite efforts to elucidate the factors related to oocytes quality at the molecular level, it remains technically difficult to develop an index based on the visualization of these factors. Thus, this article reviews the current literature on different non-invasive markers that have been correlated with oocyte quality in mammalian species, with emphasis on the bovine model, and exploring the utility of each grading system.

2. Non-invasive markers for the selection of the best oocytes

2.1. Ovarian morphology

During the retrieval of oocytes from slaughterhouse material, the collection of ovaries based on the presence or absence of estrus cycle structures, i.e. presence or absence of follicles and corpus

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luteum (CL), have been used as a straightforward non-invasive criterion to access developmentally competent oocytes. However, there are discrepancies among different studies in this regard. Early studies indicated that the presence of a dominant follicle (>10 mm) inone or both ovaries had a negative effect on *in vitro* developmental competence of oocytes derived from the subordinate follicles [15-17]. Manjunatha et al. [18] reported that oocyte development was maximal in ovaries with a CL and no dominant follicle, whereas those with a CL and a dominant follicle showed maximum development only when oocytes were derived from the dominant follicle. In agreement with this notion, Pirestani et al. [19] reported that oocytes derived from ovaries containing a large follicle (~20 mm) were less competent compared to embryos derived from ovaries that containing a CL. Similarly, Penitente-Filho et al. [20] classified cumulus-oocyte complexes (COCs) under the stereomicroscope and indicated that ovaries with CL yielded a greater number of good quality oocytes than ovaries without CL. However, the oocytes used in the latter study were not subjected to IVP to confirm their developmental competence. Overall, these studies indicate that the presence of a dominant follicle in the bovine ovary has a negative impact on subsequent embryo development, while the presence of a CL favors oocyte competence. In contrast, more recent studies indicate that the presence of a CL has negative effects on the developmental competence of ipsilateral oocytes [21,22]. However, this ''negative'' effect does not influence the competence of oocytes originated from large follicles (10-20 mm) as much as those derived from small and medium follicles (<9 mm) [22].

Ovaries without structures indicative of estrus cyclicity have less competent oocytes than others [18,23], as indicated by the presence of fewer than 10 follicles 2 to 5 mm in diameter and no large follicles [24]. In addition, other authors have indicated that the developmental competence of bovine oocytes from antral follicles (2 to 8 mm) is not affected by either the presence of a dominant follicle or the phase of folliculogenesis [24-28]. Thus, despite the few discrepancies, it seems that the selection of ovaries based on the presence of cycle-related structures could help optimize access to oocytes with better developmental competence for *in vitro*-derived technologies. Nevertheless, the positive or negative effects of ovarian structures on oocyte competence require further investigation to determine more precisely how these ovarian structures impact subsequent *in vitro* embryonic development.

2.2. Follicle size

One of the most used criteria to obtain competent oocytes is the size of the follicle. Research over the past decades indicates that bovine oocytes acquire developmental competence late in the follicular phase, when signs of atresia are observed for the first time, such as a slight expansion in the outer cumulus layers and some cytoplasmic granulations [7,29]. Therefore, the recommendation is that oocytes recovered from follicles between 6 and 10 mm develop more frequently to more advanced embryonic stages [7,30-33]. Although the acquisition of competence begins when the follicle reaches 3 mm and the effect of size becomes more important at 8 mm [16,34,35], success is not guaranteed even if the oocytes come from larger follicles [Reviewed by 36].

The acquisition of oocyte competence seems to be due to the substrate support received and to the developmental phase at the time of removal from the follicle. Recent reports indicate that the follicular fluid microenvironment of large follicles has higher levels of electrolytes, glucose, reactive oxygen species, glutathione, superoxide dismutase activity, lipids, cholesterol, pyruvate, and oestradiol [30,37,38]. Moreover, oocytes derived from larger follicles also show a different transcriptional pattern for chromatin remodeling and metabolic pathways, such as lipid metabolism, cellular stress and cell signalling, than those coming from smaller sizes, which would favor their developmental potential [38,39]. Therefore, these findings indicate that large follicles (> 6 mm) provide an appropriate microenvironment for the oocyte leading to better embryonic development.

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2.3. Morphology of the cumulus-oocyte complexes

The quality of COCs can be influenced by multiple factors, both intrinsic and extrinsic. Intrinsic factors include breed, age, reproductive status, metabolic and nutritional status, hormonal levels and stage of the estrous cycle [Reviewed by 40], whereas the main extrinsic factors include the time between slaughter and extraction of COCs, morphology and methods of collecting the COCs, storage temperature of the ovaries, collection media and the ability of the operator [41].

Since intrinsic factors are more difficult to control when using slaughterhouse ovaries from cows of unknown origin, the morphology of the COC is relatively easy to evaluate and is often the most common criterion used to select and classify a standard collection of bovine oocytes [42-44]. Morphological criteria include the number of layers of cumulus cells, their appearance, and the cytoplasmic features of the oocyte, such as the texture or brightness of its cytoplasm. Basically, the healthiest COC quality (Class I) relates to a complete cumulus cover with several compact cell layers; medium quality (Class II) has only partial cumulus cover and/or slightly expanded cumulus containing less than five cell layers; and finally, the worst quality (Class III) has darker cytoplasm and the presence of dark spots with expanded cumulus, all indicative of follicular atresia. However, such classification criteria vary among laboratories.

The study by Wit et al. [27] classified COCs into three groups: i) compact and bright, ii) less compact and dark, and iii) strongly expanded cumulus with dark spots, where developmental capacity, measured by *in vitro* embryo production, was correlated with COCs appearance. Moreover, less compact and darker COCs showed faster meiotic resumption. Another study using similar categories reported that COCs with darker cumulus and ooplasm were the most competent in terms of cleavage and blastocyst production after *in vitro* fertilization and parthenogenetic activation [45]. In addition, this study also showed that developmental competence was related to calcium currents in the plasma membrane and calcium stores in the cytoplasm of immature oocytes [45]. The report by Bilodeau-Goeseels et al. [46] divided COCs into six classes based on their cumulus investment and the texture of the ooplasm. These authors found that although oocytes with fewer than five layers of cumulus cells showed lower cleavage rates, their developmental potential to the blastocyst stage was similar to oocytes with more than five layers of cumulus cells. More recently, De Bem et al. [34] found that class III COCs, considered to be of poor morphological quality, were superior in terms of blastocyst development to the intermediate class II group, but similar to class I COCs, although without differences in blastocyst quality. Emanuelli et al. [47] indicated that COCs with partial (less than five cell layers) and expanded cumulus had higher levels of DNA fragmentation after IVM and lower competence compared to healthier ones, in accordance with the report by Yuan et al. [48]. However, blastocysts derived from COCs with different morphological classifications exhibited no variations in terms of quality assessed by the number of cells. In addition, Emanuelli et al.[47] further concluded that these differences were due to better nuclear maturation through enhanced maintenance of metaphase II (MII) block by COCs showing full cumulus coverage.

Thus, despite these contradictory results, most studies agree that COCs showing signs of early atresia yield high blastocyst rates compared to morphologically healthy COCs. Nonetheless, advanced atresia such as the presence of granulations, less than five layers of cumulus cells, absence of cumulus, or the presence of expanded cumulus with dark clumps, show lower *in vitro* potential as measured by cleavage rates and blastocyst formation [27]. Additionally, although morphological classification seems to influence the proportion of blastocysts formed, such criteria may not influence their quality. Therefore, when selecting COCs based on their cumulus investment and ooplasm texture, at the ideal would be to target COCs with several cumulus cell layers (more than or at least five layers), compact and/or slightly expanded, with or without dark areas in the oocyte and cumulus.

2.4. Lipid content

The morphological appearance of the ooplasm of immature oocytes, which is commonly used as a quality parameter [49,50] is influenced by lipid content in livestock species, such as cattle, pigs and horses [51-53]. Lipids, in the form of lipid droplets (LD), are important signaling molecules involved in regulatory mechanisms of oocyte maturation and competence acquisition [Reviewed by

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54]. In the late stage of oocyte maturation and during pre-implantation development, endogenous oocyte lipids work as an energy source [55,56] and as a lipid factory for energy reserve [57]. Failure to use lipids in oocytes has been shown to be related to inadequate nuclear maturation [58,59]. The number of LD present in the cytoplasm increases as the oocyte grows [60] and, although the ooplasm organization does not undergo major changes during *in vitro* maturation to MII [53], the type and amount of lipids in the LD seem to be more dynamic and to undergo changes during meiotic progression to MII [56,61].

LD droplets aggregate in the form of dark clusters that can be seen in the ooplasm as a cytoplasmic darkness [52,62]. Cytoplasmic darkness can be homogeneous, affecting the entire cytoplasm or concentrated in the center, with a clear peripheral ring that gives the cytoplasm a darkened appearance. This opaque appearance is more intense in pigs and domestic cats, followed by cows and finally sheep and goats, whose ooplasm is lighter. In the case of horses, lipid polarization is commonly observed, which facilitates the visualization of the spermatozoon within the oocyte [52,63].

Several studies have investigated the relationship between oocyte lipid content and competence. For instance, cytoplasm color can be used as an indicator of the lipid content of oocytes and to predict embryo development efficiency [64], as oocytes with a uniform and brown or dark cytoplasm contain more intracellular lipids than oocytes with a granular or pale cytoplasm [62]. Most studies have demonstrated that the culture of immature oocytes with a coarsely granulated or very pale ooplasm results in lower blastocyst yields [46,50,64]. Jeong et al. [65] classified the ooplasm in three categories: dark, brown, and pale. In this study, the content of mitochondria and the proportion of oocytes that cleaved and developed to the blastocyst stage were higher in the darker group. Also, Nagano et al. [64] reported that sperm penetration, normal fertilization, cleavage and blastocyst rates were higher in oocytes in which the cytoplasm appeared brown, compared with pale or very dark ones. Moreover, brown oocytes with a dark zone at the periphery or with dark clusters showed under electron microscopy an organelle arrangement similar to *in vivo* matured oocytes, and pale or black oocytes appeared to be degenerating and/or aging. The authors concluded that a dark ooplasm indicates an accumulation of lipids and better developmental potential, while a pale ooplasm indicates a low density of organelles and poor developmental potential [Reviewed by 66]. Interestingly, a study by Prates et al. [67] distinguished fat areas of different color shades using the Nomarski interference differential contrast (NIC) as the fat gray value of porcine oocytes, reflecting alterations in lipid content, and proposing this tool as an appropriate and non-invasive technique to evaluate the lipid content of a single oocyte before or after *in vitro* maturation.

Taken together, and as stated by the review of Nagano and colleagues [66], a dark ooplasm indicates an accumulation of lipids and good developmental potential, light-colored ooplasm indicates a low density of organelles and poor developmental potential, and black ooplasm indicates aging and low developmental potential. Finally, the use of NIC should be further investigated as a potential non-invasive tool to evaluate the lipid content of single oocytes in other livestock species.

2.5. Cumulus expansion and oocyte size

Another parameter that is often used as an indirect indicator of oocyte quality is the degree of cumulus expansion following maturation, typically after 20 to 24 h of culture in an *in vitro* maturation environment. Grades 1 to 3 (sometimes 4) are attributed to increasing degrees of expansion (1: poor expansion, characterized by few morphologic changes compared to before maturation; 2: partial expansion, characterized by little expansion but notable clusters lacking expansion and 3: complete or nearly complete expansion) [68-70].

Although the expansion of cumulus cells has been described as the basis for oocyte maturation [71] and early reports supported the idea that quantity and quality of the expanded cumulus mass were correlated with developmental capacity [72], its usefulness as an indicator of developmental potential in bovine seems to be modest [73]. For instance, studies by Anchordoquy et al. [74], Dovolou et al. [75] and Rosa et al. [76] reported that, under different experimental conditions, the cumulus expansion index was not indicative of blastocyst yield or quality. Similarly, another study indicated

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that inhibition of cumulus expansion by enzymatic hyaluronidase degradation did not affect cleavage or blastocyst development [77]. Nonetheless, as showed by Fukui et al. [78], more than an indicator of developmental competence, cumulus cells and their expansion play an important role in fertilization by inducing the acrosome reaction and, therefore, promoting higher fertilization rates.

In addition to follicle size, oocyte size has also been used as a non-invasive quality parameter. Although it is difficult to measure the precise diameter of the oocyte during IVF, oocyte selection based on diameter can be used as a routine step during micromanipulation protocols. The study of Fair et al. [79] classified the oocytes recovered from slaughterhouse ovaries into four groups (< 100 microns, 100 to 110 microns, 110 to 120 microns, and > 120 microns). Rates of resumption of meiosis to MII were higher for oocytes > 110 microns. Moreover, oocytes < 110 microns were transcriptionally active, suggesting that they were still in the growth phase of oogenesis [79,80]. Similarly, Anguita et al. [81] reported that cleavage and blastocyst rates were higher in oocytes > 110 microns. Moreover, Otoi et al. [82] and Arlotto et al. [26] found that oocytes >115 microns had better rates of nuclear maturation and lower incidence of polyspermy after IVF, but cleavage rates and development to the blastocyst stage were optimal in oocytes > 120 microns. Huang et al. [83] and Yang et al. [84] compared oocytes derived from early antral follicles (0.5-1 mm in diameter) cultured *in vitro* for 14-16 days with oocytes collected from antral follicles (2–8 mm in diameter), cultured and submitted to IVM. The authors reported better maturation rate for oocytes > 115 microns and optimal for oocytes > 120 microns, but developmental competence was only high for oocytes collected from antral follicles and of size > 120 microns.

These results suggest that bovine oocytes acquire meiotic competence with a diameter of 115 microns, but full developmental competence is acquired around 120 microns, possibly because smaller oocytes have not yet completed their growth phase [43]. Thus, the selection of follicles between 6 and 10 mm, and oocytes diameters >115 and < 130 microns has the potential to optimize developmental outcomes.

2.6. First polar body assessment

At the end of IVM and after the removal of cumulus cells, it is easy to perform a detailed observation of morphological features [10], including the assessment of oocyte shape, cytoplasm color and granulation, regularity and thickness of the zona pellucida, size of the perivitelline space, presence of vacuoles, and presence or absence of the first polar body (PB1) and its morphology. Extrusion of the PB1 in mammalian oocytes is a cellular landmark of meiotic maturation and its assessment is frequently used as an indicator of nuclear maturation [85]. Thus, its absence indicates that the oocyte is immature or that it has degraded due to aging but also its presence does not guarantee that the oocytes have completed their maturation process and some of them remain incompetent despite exhibiting morphologic features of nuclear maturation [86].

In bovine species, extrusion of the PB1 begins at 16-18 h after IVM [87-90]. Nonetheless, oocytes acquire the highest developmental competence at around 5 to 10 h after PB1 extrusion [11,91]. Dominko and First [91] indicated that oocytes that extruded their PB1 after 16 h of IVM required another 8 h of culture to reach higher developmental capacity. Thus, cytoplasmic maturation in cattle occurs several hours after nuclear maturation, probably between 24 and 30 h after the beginning of IVM.

Unfortunately, there are no studies that analyze the influence of the first PB morphology on oocyte competence in cattle. However, one study using porcine oocytes indicated that PB1 with a smooth or intact surface was indicative of a more advanced cytoplasmic maturation and better embryonic development *in vitro* than those with a fragmented or rough surface [92]. Despite lacking studies in domestic species, studies in humans have investigated the correlation between PB1 morphology and oocyte competence [93,94]. Ebner et al. [95] conducted a retrospective study using 70 consecutive ICSI cases in which oocyte classification based on PB1 morphology revealed that oocytes with intact, well-shaped PB1 yield higher fertilization rates and higher quality embryos. Later, Ebner et al. [93] confirmed the relationship between PB1 morphology, fertilization and blastocyst quality, but also a positive effect on implantation and pregnancy rates. Similarly, Rose et

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al. [96] reported that oocytes with an intact first PB have high fertilization rates and better embryonic development, whereas those displaying a PB1 of large size, irregular shape, rough surface or fragmentation are less competent after IVF, yielding low pregnancy rates after embryo transfer. In contrast, others have not reported any correlation [97-99]. Thus, there is a lack of consensus on the impact of PB1 morphology on oocyte competence and embryonic development in humans. It is also important to note that some PB1 abnormalities may be an artifact of oocyte manipulation (mainly during the denudation process) or aging [100].

In summary, although the selection of oocytes with first PB1 of a homogeneous, round shape, smooth or intact surface may be indicative of a better oocyte, the usefulness of this selection criterion in livestock requires further research to establish its real predictive value for oocyte competence.

2.7. Meiotic spindle and zona pellucida birefringence

Polarized light microscopy (PLM) allows non-invasive evaluation of the meiotic spindle of metaphase oocytes in different animal species. To learn about the principles and equipment required for PLM in detail, readers are directed to excellent reviews on the subject [101,102]. Using PLM, it is possible to locate and evaluate the morphology of the meiotic spindle to confirm egg maturation, which has been positively correlated with developmental competence [86,103-105]. This method avoids damaging the spindle during the ICSI procedure, considering that the position of the PB1 can be altered when cumulus cells are removed during preparation for ICSI [106]. Also, PLM has been successfully used to remove the meiotic spindle and chromosomes (enucleation) in mice [107], bovines [108], and pigs [109], with an average efficiency of 90% and, more importantly, avoiding the exposure to UV rays and their detrimental effect on embryonic development.

In livestock species, the dark appearance of the ooplasm, attributed to high lipid contents, is known to interfere with spindle imaging [109] and, as in humans, precludes the detection of meiotic spindle abnormalities [98,109,110]. Therefore, spindle birefringence should be carefully considered as an indicator of oocyte quality and chromosome alignment in some species. In pigs, a negative PLM signal was associated with to reduced maturation and poor development potential [109]. In the same study, when the PLM system was used for spindle removal, the overall enucleation efficiency was 92.6%, indicating that PLM is an effective tool for performing enucleation in pigs. A few years later, the same group evaluated the use of PLM to assess the meiotic spindle of *in vitro*-matured bovine oocytes after vitrification and warming [111]. They were able to confirm the presence of the meiotic spindle in 99% of the analyzed eggs. Moreover, after vitrification and warming, meiotic spindles were detected in 79% of oocytes. Interestingly, thawed oocytes that displayed a positive PLM signal showed better competence in terms of cleavage and blastocyst rates after parthenogenetic activation, indicating that PLM can be a useful tool for assessing post-warming viability in vitrified bovine oocytes.

Overall, these studies demonstrate that polarized light microscopy is an effective system to detect the meiotic spindle in livestock oocytes and does not exert a detrimental effect on preimplantation developmental competence. However, the selection of oocytes by the presence of a PLM signal does not seem to offer improvement in IVP outcome in cattle.

In addition, PLM has been used for the evaluation of the zona pellucida birefringence (ZPB) which in humans has been correlated with oocyte quality [112,113]. The few studies in cattle have shown that a lower ZPB is related to high quality oocytes and improved blastocyst development [114,115], whereas two studies in horses have reported conflicting results, indicating beneficial effects of both low ZPB [116] and high ZPB [117]. Because most of the studies with PLM have been carried out in mice and humans with conflicting results, its potential application and practical use in cattle and other livestock species needs further assessment. Contrary to the work in humans that uses a limited number of highly valuable oocytes from infertile and/or older women, livestock oocytes obtained from slaughterhouse ovaries allow a more stringent selection. Furthermore, assessment of the meiotic spindle is a time-consuming process, which delays the overall process of *in vitro* embryo production. Routine application in reproductive biotechnology would require PLM to show a clear advantage and improvement over conventional approaches using oocyte morphological selection

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criteria. However, PLM might be beneficial when individual oocytes are of high value, such as oocytes recovered from elite cows by ovum-pick-up (OPU) or when manipulation of only a few oocytes is required, i.e. SCNT and ICSI [107,109].

2.8. Brilliant cresyl blue (BCB) staining

Another approach of proven predictive value is to assess glucose-6-phosphate dehydrogenase (G6PDH) activity by brilliant cresyl blue (BCB) staining. BCB is a dye that determines the intracellular activity of glucose-6-phosphate dehydrogenase (G6PDH). Activity of G6PDH is observed during the oocyte growth phase (BCB-: colorless cytoplasm, increased G6PDH) due to the demand of ribose-6-phosphate for nucleotide synthesis. This activity is low (BCB+: colored cytoplasm, low G6PDH) in oocytes that have completed their growth phase [118]. This technique has been successfully used as a diagnostic tool for oocyte evaluation in various species, including cattle [118-120].

Although previous reports found that the developmental competence of oocytes with low G6PDH activity (BCB+) was higher than that of oocytes with a high G6PDH activity (BCB-), the absence of significant differences between the blastocyst rates developed from BCB+ and the untreated control group, decrease the usefulness of the BCB test in IVP technology [Reviewed by 121]. However, it is unquestionable that BCB+ oocytes have statistically higher developmental competence than BCB- oocytes, both in IVF and somatic cell nuclear transfer (SCNT) [Reviewed by 121].

Later studies continued to show only trend of BCB+ oocytes towards greater developmental potential. Better blastocyst rates at day 7 were reported by Silva et al. [122], and a study by Fakruzzaman et al. [123] reported higher blastocyst quality based on total, apoptotic cells, and mitochondria numbers. Similarly, Castaneda et al. [124] indicated that the higher lipid content of BCB+ bovine oocytes might provide a cellular and functional basis for their better developmental competence. Interestingly, another article indicated that, using BCB+, the co-culture of competent oocytes with non-competent oocytes during IVM reduces their capacity to undergo embryonic development and reach the blastocyst stage [125]. However, others have confirmed that not all BCB+ oocytes will lead to perfect embryonic development and that the BCB test is not sufficient for the identification of more competent oocytes [126]. Nonetheless, the combination of oocyte and zygote selection using the BCB test would enhance the efficiency of high-quality embryos selection, compared to the single BCB test [127]. Therefore, the BCB test can be a valuable tool when used together with classical morphological classification and could be useful for the selection of oocytes with a higher implantation potential.

3. Conclusions and future perspectives

The classification and selection of oocytes in livestock species for *in vitro* embryo production and for micromanipulation techniques, such as ICSI and SCNT, can be one of the most important steps to reach superior embryonic development and quality. Additionally, the timing of fertilization can influence the final outcome, especially for ICSI, where most sperm are injected between 20 and 24 h after IVM, while during IVF sperm penetration can occur later. Furthermore, studies that perform embryo transfers are also important to effectively evaluate developmental potential, as successful embryo implantation is highly dependent on the quality of the embryo and the intricate relationship it establishes with the uterine endometrium. Ultimately, with the advent of bovine embryonic stem cells, greater scrutiny of oocytes with high developmental potential is necessary, for the production of stable pluripotent stem cell lines to be used in basic science, forward and reverse genetics, epigenetics, gene imprinting, as well as in the production of animal models with applications in animal production. Thus, besides improving the conditions to support *in vitro* maturation, the implementation of new tools for the assessment of gamete competence, together with studies decoding molecular cues in oocyte maturation, will improve our understanding of this complex process and will more precisely identify the synchrony between nuclear and cytoplasmic maturation in livestock species

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