Developmental Competence of Bovine Oocytes Selected by Brilliant Cresyl Blue Staining: Effect of the Presence of Corpus Luteum on Embryo Development

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Abstract: *The local circumstances of the ovary may influenced by the presence of corpus luteum (CL). We investigated the cleavage rate and blastocyst development of bovine oocytes collected from ovaries with or without CL. Oocytes were incubated with brilliant cresyl blue (BCB) for 90 min and separated on the basis of low (BCB+) or high (BCB–) activity of glucose-6-phosphate dehydrogenase. For both types of ovaries, the embryo cleavage rates in the case of BCB+ oocytes were significantly higher than those in the case of BCB– oocytes, but were not higher than the rates in the control group. The percentage of blastocysts developing from BCB+ and BCB– oocytes from the ovaries with CL did not significantly differ. However, in the case of ovaries without CL, a significantly higher ($P<0.05$) percentage of blastocysts developed from BCB+ oocytes than from BCB– and control group oocytes. The presence or absence of CL did not significantly influence the cleavage rate and blastocyst development. Based on our results, we conclude that BCB staining facilitates the selection of competent oocytes that will develop into blastocysts for IVF better than conventional morphological selection methods. We also conclude that the CL does not significantly influence blastocyst development.*

Key words: *Bovine, BCB, Oocytes, Corpus luteum*

Introduction

In *in vitro* production of bovine embryos, the immature oocytes are heterogeneous in terms of quality and developmental competence [1] because the origin of the ovaries and the reproductive status of the animals are usually unknown. Bovine oocytes capable of normal development show morphological variation, and morphological assessment has led to some improvements in the identification of oocytes suitable for development *in vitro* [2]. Oocyte morphology can indicate maturity and fertilization potential [3–5]. Nevertheless, even after careful selection on the basis of visual appearance, less than 30% of immature bovine oocytes recovered from slaughterhouse ovaries develop into blastocysts after *in vitro* culture (IVC). This is probably due to the quality of the oocytes at the beginning of maturation, and indicates the need for the development of other approaches. Brilliant cresyl blue (BCB) stain facilitates the selection of competent oocytes for *in vitro* embryo production. The use of BCB stain has been evaluated in pigs [6, 7], goats [8], and bovine oocytes [9–12]. However, these studies did not evaluate the effect of the presence or absence of the corpus luteum (CL) in the ovary on the cleavage rate and blastocyst development after BCB staining. The ability of oocytes to develop can be assessed *in vitro* in the presence of glucose-6-phosphate dehydrogenase (G6PD). This enzyme is synthesized within the oocytes during the growth phase. Its activity decreases once the growth phase is completed and the oocytes achieve developmental competence [13, 14]. BCB dye is degraded by G6PD. In bovine *in vitro* fertilization (IVF), the results of BCB staining indicated that oocytes with a completed growth phase and those still growing have different levels of G6PD [15]. This is explained by the fact that the BCB stain reflects the activity of G6PD, an enzyme that is actively synthesized in growing oocytes (BCB–) but is less active in fully grown oocytes (BCB+).

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In bovine IVF and *in vitro* culture, the conventional method for selecting cumulus-oocyte complexes (COCs) before maturation relies on follicular size. However, the selection of oocytes based on this criterion appears to be inadequate because follicles with identical diameters from the same individual can have quite dissimilar physiological phases. Additionally, even follicles from the same ovary [16–19] that have identical diameters may exhibit different physiological phases. This depends on many factors, including the presence or absence of CL, transportation time, and the stage of development of the oocytes at the time of collection. Although some improvement has been made regarding the selection of COCs by using BCB for predicting oocyte competence in IVF, the influence of ovarian morphology and selection of oocytes based on the BCB staining have not yet been completely investigated. Therefore, the purpose of this study was to investigate the cleavage rate and blastocyst development of oocytes collected from ovaries with or without CL after selection by BCB staining.

Materials and Methods

All chemicals were tested for use in cell culture and were purchased from Sigma, unless specified, otherwise.

Classification of the ovaries

Bovine ovaries were obtained from a slaughterhouse and were transported to the laboratory in a thermoflask containing saline (0.85% NaCl) at 26°C within 8 h of slaughter. The ovaries were thoroughly washed in saline and classified based on the presence of active, red CL or the complete absence of CL. Ovaries with regressed CL or yellowish CL were excluded from all the experiments.

Collection of COCs

COCs were collected separately by aspiration from the antral follicles (2–6 mm diameter) by using an 18-gauge needle connected to a 5-ml disposable syringe. The aspirates were placed in 40-ml conical glass tubes for 20 min at 37°C for sedimentation. Subsequently, the supernatant was removed, and the remaining 0.5 ml of COCs was sedimented in Dulbecco's phosphate buffered saline (D-PBS) for 15 min. After removing the supernatant, the remaining 0.5 ml was transferred to a Petri dish. Oocytes possessing a full cumulus mass, unfragmented cytoplasm, and intact zona pellucida were used in the experiments.

BCB staining

The COCs were washed 3 times in D-PBS without bovine serum albumin (BSA) and exposed to 26 μM BCB (B 5388) diluted in D-PBS for 90 min at 38.5°C in a humidified atmosphere. Following exposure to BCB, the oocytes were washed 3 times in D-PBS. The COCs were examined under a stereomicroscope at 50 × magnification and classified as follows: (1) BCB+ group, oocytes with varying degree of blue cytoplasm (grown oocytes), and (2) BCB– group, oocytes with no blue cytoplasm (growing oocytes).

In vitro maturation

Control group oocytes were washed 3 times in D-PBS supplemented with 3% CS and 5 μg/ml gentamicin sulfate and 3 times in TCM-199 [20] supplemented with 0.02 mg/ml FSH and 5% CS. After washing, the COCs were matured in the same medium covered with paraffin oil (25–30 COCs/100 μl droplet) at 38.5°C under 5% CO_2 in air for 20 h.

After the classification of COCs based on the BCB staining results, the BCB exposed group were washed 3 times in D-PBS supplemented with 3% CS and 5 μg/ml gentamicin sulfate and 3 times in TCM-199 supplemented with 0.02 mg/ml FSH and 5% CS. After washing, the COCs were matured as previously described. The COCs were not exposed to BCB prior to determining the effect of the CL on the cleavage rate and blastocyst development *in vitro*.

IVF

IVF was performed using frozen-thawed semen from a single ejaculate of a Holstein bull. The frozen 0.25-ml straw was thawed at 37°C for 30 s. The thawed semen was placed on 45% and 90% Percoll [21] and centrifuged at 700 × g for 20 min. After removing the top layers, the sperm pellet was resuspended in 6 ml Brackett and Oliphant medium (BO) and washed again by centrifugation at 500 × g for 5 min [22]. Spermatozoa were counted in a hemacytometer and diluted in an appropriate volume of BO medium to yield a concentration of 5×10^6 spermatozoa/ml. After *in vitro* maturation (IVM), the oocytes were washed 3 times with BO medium, with gentle pipetting to partially remove the cumulus cells. Up to 30 oocytes from both the control and treatment groups were placed in 100 μl insemination droplets under mineral oil and incubated for 18 h at 38.5°C in 5% CO_2 in humidified air.

IVC

After IVF for 18 h, the presumptive zygotes were

removed from the cumulus cells by gentle vortexing in CR1aa [23] supplemented with 5% CS and 5 μg/ml gentamicin sulfate. The zygotes were washed 3 times in CR1aa supplemented with 5% CS. Subsequently, 30 zygotes were cultured in 30 μl CR1aa supplemented with 5% CS and 50 μg/ml of gentamicin sulfate under paraffin oil and incubated at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The cleavage rates of the embryos were examined at 48 h after IVC. The cleaved embryos were examined for blastocyst development on days 7–9 after IVF.

Blastocyst cell count

The number of cells in the blastocyst was determined using the Hoechst 33342 staining technique. The blastocysts were washed in PBS plus 2% Triton X100 for 15–20 s and stained overnight with Hoechst 33342 (25 μg/ml) and ethanol (99.5%) at 4°C. They were then embedded in glycerol, fixed on a slide, and covered with a coverslip. The number of cells per blastocyst was counted under a fluorescence microscope, with an excitation filter of 365 nm and a barrier filter of 410 nm.

Statistical analyses

Each experiment was repeated 5 times. All the data were compared by Chi-square analysis or Fisher's exact probability test.

Results

A total of 1,070 COCs were recovered from the slaughterhouse ovaries and used in the experiments. Of these, 642 COCs were used to investigate the competence of bovine oocytes in the presence of CL after selection by BCB staining and to investigate subsequent embryo development. The remaining 428 COCs were used to investigate the competence of bovine oocytes obtained from ovaries with CL and without exposure to BCB. In the case of ovaries with CL, the percentage of BCB+ oocytes 73.9% was significantly higher (P<0.05) than that of BCB– oocytes 26.1% (Table 1). Furthermore, the ovaries without CL yielded 69.5% BCB+ oocytes and 30.5% BCB– oocytes; the difference was significant (P<0.05). No significant difference in the percentages of BCB+ and BCB– oocytes was observed between the 2 types of ovaries.

Table 1. Quality of oocytes selected by BCB staining obtained from ovaries with or without CL

Status of the ovary	No. of oocytes	No. of oocytes	
		BCB+ (%)	BCB– (%)
With CL	222	164 (73.9)[a]	58 (26.1)[b]
Without CL	239	166 (69.5)[a]	73 (30.5)[b]

[a, b]Values with different superscripts within a row differ (P<0.05).

Table 2 shows the results of embryo development of the oocytes selected from ovaries with or without CL by BCB staining. The cleavage rate of BCB+ embryos obtained from the ovaries with CL was significantly higher (P<0.05) than that of BCB– embryos, but was not higher than that of embryos obtained from the ovaries without CL and that of the control group. Furthermore, the cleavage rate of BCB+ embryos obtained from the ovaries without CL was significantly higher than that of BCB– embryos (P<0.05). All blastocysts obtained from the control and BCB groups exhibited normal morphology, indicating that BCB staining had no detrimental effects on embryo development. The percentage of BCB+ oocytes that developed into blastocysts in the group from ovaries with CL (32.3%) was not significantly different from that of BCB– oocytes (25.9%). However, the percentage of BCB+ oocytes that developed into blastocysts in the group from ovaries without CL (34.9%) was significantly higher (P<0.05) than those of BCB– (20.5%) and the control group oocytes (26.5%). The number of cells in the

Table 2. Development of embryos from BCB selected bovine oocytes obtained from ovaries with CL

Status of the ovary	Group	No. of oocytes	No. of cleaved (%)	No. of blastocyst development (%)	Cell number per blastocyst
With CL	BCB+	164	117 (71.3)[a]	53 (32.3)	120 ± 7.6
Without CL	BCB+	166	121 (72.9)[a]	58 (34.9)[c]	121 ± 7.9[a]
With CL	BCB–	58	26 (44.8)[b]	15 (25.9)	104 ± 10.8
Without CL	BCB–	73	44 (60.3)[b]	15 (20.5)[d]	85 ± 16.5[b]
	Control	181	116 (64.1)[a]	48 (26.5)	117 ± 13.2

[a, b]Values with different superscripts within a column differ significantly (P<0.05).

Table 3. Effect of CL on cleavage rate and blastocyst development of *in vitro* fertilized bovine oocytes

Status of the ovary	No. of oocytes	No. of cleaved (%)	No. of blastocyst development (%)	Cell number per blastocyst
With CL	200	132 (66.0)	58 (29.0)	117.3 ± 8.3
Without CL	228	162 (71.1)	70 (30.7)	124.0 ± 7.9

blastocysts was comparable in all groups. An exception was the BCB– group from ovaries without CL, in which the number of cells in the blastocysts was significantly lower ($P<0.05$) than that in the corresponding BCB+ group.

The results of the effect of CL on the cleavage rate and blastocyst development are presented in Table 3. No significant differences were observed between ovaries with or without CL in terms of cleavage rate, blastocyst development, and the number of cells in the blastocyst.

Discussion

The developmental competence of bovine oocytes for IVM/IVF/IVC was not influenced by the presence or absence of CL in the ovaries, but BCB staining of bovine oocytes improved *in vitro* embryo production. However, the biochemical basis of BCB metabolism in COCs is not fully understood. In the present study, a large percentage of fully grown (BCB+) oocytes that were selected by BCB staining could be used for IVF (Table 1). Our results are similar to those of previous studies that used the same method. However, our results are higher than that reported by Alm *et al.* [10]. This is probably because the oocytes matured within the follicles prior to collection during the transportation of the ovaries in saline solution after the slaughter of the animals [24]. We speculate that these differences were caused by the following 2 reasons: (1) some oocytes require more than 90 min of incubation; and (2) the selection criteria after BCB staining vary among laboratories. Nuclear maturation was not affected by either exposure or non-exposure to BCB. Alm *et al.* [10] reported that a lower proportion of BCB– oocytes reached metaphase II compared to the control, holding control, and BCB+ oocytes. Similar findings have also been reported in goat oocytes [8]. In our experiment, we did not investigate nuclear maturation. In bovine IVF, morphological characteristics such as full cumulus mass, unfragmented cytoplasm, dark cytoplasm, and intact zona pellucida are routinely used to select oocytes in most laboratories. Furthermore, it is possible to achieve fertilization rates of 75–80% [25, 26] and cleavage rates of more than 70% [27–30]. In reality, however, less than 30% of recovered oocytes develop into blastocysts. This is probably due to the quality or competence of the oocytes at the beginning of maturation.

In our study, both the cleavage and blastocyst development rates were influenced by the selection of oocytes using the BCB staining test before maturation. Our results are consistent with those of previous studies [9, 12] indicating that exposure to BCB solution has no detrimental effect on oocytes, even when BSA supplementation was not performed. In contrast, a previous study [10] reported that cleavage rates in the BCB+ and BCB– groups were identical, but the blastocyst rate was very low in the latter group. The reasons for this discrepancy are unclear. The mean number of cells in the blastocysts of all groups were comparable, except for the BCB– group obtained from the ovaries without CL. In this group, fewer cells were seen than in the BCB+ group obtained from the same type of ovaries (Table 2). This result is consistent with the results of previous studies [10, 31–33]. However, our results regarding the number of cells in the blastocysts are higher than those obtained by Pujol [9], who reported blastocyst cell numbers from BCB+, BCB–, and control oocytes.

No changes were observed in the cleavage and blastocyst development rates of bovine IVF oocytes (Table 3), and our results are consistent with those of previous studies [34–36]. However, a few studies [37] have reported higher cleavage rates in ovaries with CL than in ovaries without CL. Machatkova *et al.* [38] observed higher blastocyst production rates in bovine oocytes recovered on days 14–16 of the estrus cycle than on days 7–9, which in turn influenced developmental competence *in vitro*. The presence of CL probably increases the viability of bovine embryos. The reasons for these differences in blastocyst development rates are presently unknown. This may be due to a high circulatory progesterone level, which

results in the regression of dominant follicles and emergence of constant follicle-wave turnover [39, 40]. However, the wave pattern in follicular development or intrafollicular environment to which the oocytes are exposed may be factors that irregularly affect the achievement of competency by bovine oocytes. Oocytes collected from ovaries with and without CL may vary depending on the stage of the estrus cycle, ovarian condition, and follicular size.

There is increasing evidence that germinal vesicle breakdown commences in subordinate follicles in the early regression phase and in the late static phase in the absence of the preovulatory LH surge [41]. Thus, oocytes in this phase would show better embryonic development than the oocytes in earlier phases. Ginther *et al.* [42] concluded that the effects of follicular suppression are wrought by the dominant follicle via systemic rather than local channels. The development of small antral follicles through the intra-ovarian effect of CL has been reported in cattle [43]. Previous studies have also shown that compared to ovaries without CL, those with CL exhibit improved follicular growth during the luteal phase as well as good follicular development [44]. The development of oocytes into embryos after IVF from anestrus and pregnant cows is poor, but these oocytes are sufficiently developed to be considered for use in *in vitro* production. Another study [45] reported almost identical cleavage rates of oocytes collected from pregnant and non-pregnant cows. In our study, no significant difference was observed in the developmental competence of oocytes obtained from ovaries with and without CL even after selection by the BCB test. This finding is consistent with the results of the above-mentioned studies. Although the oocyte quality is not affected by CL, it appears that follicles in an ovary somehow influence each other. In conclusion, based on our results, we conclude that the BCB staining test of bovine COCs facilitates the selection of a greater number of competent oocytes that can develop into blastocysts for use in IVF than can be obtained via conventional morphological selection methods. Further, the BCB staining test does not affect the blastocyst quality. We also conclude that the presence of CL in the ovary has no significant influence on oocyte quality.

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