

Research Article

Selection of Ovine Oocytes by Brilliant Cresyl Blue Staining

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Sheep oocytes derived from the ovaries collected from the slaughterhouse are often used for research on *in vitro* embryo production, animal cloning, transgenesis, embryonic stem cells, and other embryo biotechnology aspects. Improving the *in vitro* culture efficiency of oocytes can provide more materials for similar studies. Generally, determination of oocyte quality is mostly based on the layers of cumulus cells and cytoplasm or cytoplasm uniformity and colors. This requires considerable experience to better identify oocyte quality because of the intense subjectivity involved (Gordon (2003), Madison et al. (1992) and De Loos et al. (1992)). BCB staining is a function of glucose-6-phosphate dehydrogenase (G6PD) activity, an enzyme synthesized in developing oocytes, which decreases in activity with maturation. Therefore, unstained oocytes (BCB⁻) are high in G6PD activity, while the less mature oocytes stains are deep blue (BCB⁺) due to insufficient G6PD activity to decolorize the BCB dye.

1. Introduction

Sheep oocytes derived from the ovaries collected from the slaughterhouse are often used for research on *in vitro* embryo production, animal cloning, transgenesis, embryonic stem cells, and other embryo biotechnology aspects. Improving the *in vitro* culture efficiency of oocytes can provide more materials for similar studies. Generally, determination of oocyte quality is mostly based on the layers of cumulus cells and cytoplasm or cytoplasm uniformity and colors. This requires considerable experience to better identify oocyte quality because of the intense subjectivity involved [1–3].

Mangia and Epstein [4] and Knobil and Neill [5] found that glucose 6-phosphate dehydrogenase (G6PDH) shows activity during mice oocyte growth. With the growth of oocytes, significant changes in G6PDH occur. In addition, BCB stains the cytoplasm blue or colorless depending on G6PDH activity [6, 7]. Research in pigs [7–9], goats [10–12], cows [13–16], mice [17], and dogs [18] indicate that the percentage of BCB⁺ oocytes reaching the blastocyst stage is significantly higher than BCB⁻ oocytes. To our knowledge, there are no reports regarding the *in vitro* developmental competence of sheep oocytes selected using BCB staining.

With regard to the relationship between oocyte diameter and the ability of oocytes to develop in cows, Otoi et al. [19] found that although smaller diameter oocytes are able to undergo meiosis, they are not equipped with the developmental competence to reach the blastocyst stage. Only when the diameter of the oocyte reaches a certain threshold will its *in vitro* developmental competence become sufficient. In a study of the relationship among porcine oocyte quality, follicular size, and oocyte size, Kim found that oocytes with larger diameters achieve higher developmental competence after nuclear transfer [20]. Similarly, the same result was derived from studies on goats [21]. Hence, in this study, the diameter of the sheep oocytes was used to certify the oocytes selected using BCB.

In studies on mice, pig, cattle, and so forth [22–26], glutathione (GSH) affects cytoplasmic and nuclear maturation of oocyte, and regulates oogenesis, fertilization, and embryonic development. The GSH concentration in oocytes increases as ovulation approaches. In addition, oocytes at the MII stage have relatively higher GSH levels than those at the GV stage. After fertilization, GSH concentration declines significantly. Thus, the GSH content in oocytes reflects the maturity of the cytoplasm, and lower levels of GSH will

TABLE 1: The sequence of primers for quantitative real-time PCR.

Genes	Gene sequence (5'–3')	Fragment (bp)
<i>Mater</i>	Forward: GCTGGAGGCGTGTGGACTG; Reverse: GGTCTGTAGATTAGAGGTGGGATGC	168
<i>Zar1</i>	Forward: TGCCGAACATGCCAGAAG; Reverse: TCACAGGATAGGCGTTTGC	168
<i>Gdf9</i>	Forward: CCCAGGATTACAAGGAAG; Reverse: CTGGATGTAACCTCGACGTCTCT	198
<i>18s</i>	Forward: GAGAAACGGCTACCACATC; Reverse: GCTATTGGAGCTGGAATTAC	198

reduce the developmental competence of oocytes during *in vitro* maturation.

Liao et al. [27], Tong et al. [28], and Paradis et al. [29] found that growth differentiation 9 (*GDF9*), *mater*, *zar1*, and other maternal genes present different expressions at different stages of development in oocytes and embryos. In the laboratory, we showed that the expressions of *GDF9*, *mater*, *zar1*, and other maternal genes in oocytes and embryos are the most significant at the GV stage. Oocytes in MII stage and 2-cell to 16-cell stage embryos present low expression of the genes [30]. Therefore, oocyte maturation can be measured through the expression of *GDF9*, *mater*, *zar1*, and other genes.

In previous studies that used BCB for selecting oocytes, an *in vitro* culture channel was generally highlighted to verify its effectiveness. To our knowledge, this paper is the first report that verified the effectiveness of BCB for oocyte selection in sheep through measurement of oocyte diameter, GSH levels, and maternal gene expression.

2. Materials and Methods

2.1. Oocyte Collection. Sheep ovaries were obtained from a local abattoir and transported in sodium chloride solution containing 1000 IU/mL penicillin and 1000 IU/mL streptomycin at 30°C. At the laboratory, the ovaries were washed three times in sodium chloride. The oocytes were aspirated from follicles with the diameter of 2–6 mm using a 20-gauge needle attached to a 10 mL syringe containing 1 mL of aspiration medium [TCM-199(B-2520, Sigma) supplemented with 1 mg/mL polyvinyl alcohol (P-1763, Sigma) and 29 µg/mL heparin sodium salt(H-3393, Sigma)]. Only oocytes with complete cumulus layers and homogeneous cytoplasm were selected for use.

2.2. Brilliant Cresyl Blue Staining Test. Immediately after oocyte collection, they were washed three times in Dulbecco's PBS (GIBCO) modified with the addition of 0.5% (w/v) BSA (mPBS). Then, the oocytes were exposed to BCB (B-5388, Sigma) diluted in mPBS for 90 min at 38.6°C in a humidified atmosphere, washed twice, and classified into two groups, according to their cytoplasm coloration. Oocytes with or without blue cytoplasmic coloration were designated as BCB+ and BCB–, respectively. COCs of the control group were incubated directly after selection without exposure to BCB for 90 min.

2.3. In Vitro Maturation of Oocytes. After oocyte classification, the oocytes were washed three times in the

maturation medium [TCM-199 supplemented with 10% FBS (v/v), 0.05 IU/mL FSH, 0.05 IU/mL LH, 1 µg/mL 17β-estradiol, 24.2 mg/L Sodium pyruvate, 0.1 mM cysteamine, and 10 ng/mL EGF]. Oocytes were transferred in groups (20–30/drop) into 50 µL droplets of maturation medium and incubated for 24 h at 38.6°C in a humidified 5% CO₂ atmosphere under mineral oil (M-3516, Sigma).

2.4. RNA Extraction and RT-PCR. Total RNA was isolated using an RNeasy Micro Kit (Qiagen, Europe) according to the manufacturer's instructions.

RT-PCR condition: to mix 1 µg total RNA and 1 µL random six polymer primer, RNase-free water is made up to 14.5 µL at 70°C for 10 min, and experiences ice bath for 10 min. Upon the transient centrifugation, 0.5 µL RNA Inhibitor, 5 × buffer 2 µL, 10 mmol/L dNTP 2 µL, and AMV 1 µL (TaKaRa) are added in for 42°C for 40 min.

2.5. Standard Curve. *Mater*, *Zar1*, and *GDF9* genes are, respectively, connected with 18S rRNA and pMD20-T carrier. The restricted enzyme EcoR V is applied to linearize the plasmids of each gene, and linearized plasmid of each gene is undertaking dilute concentration so as to make standard curve.

According to the gene sequence in GeneBank, Oligo 6.0 software is used to design quantitative primers of ovine *Mater*, *Zar1*, and *GDF9* gene and the internal reference gene *18s*-rRNA (Table 1).

2.6. Quantitative RT-PCR. Quantitative RT-PCR (Roche, 1.5 LightCycle) reaction condition: for 2 µL standard plasmid and the sample cDNA, each 5 pM upstream and downstream primers are used, also including 2 × FastStart DNA Master SYBR Green I 10 µL (QIAGEN), plus RNase-free water to 20 µL. Reaction conditions: 95°C predenaturation for 10 min; 55 cycles include 95°C 10 s, 60°C 20 s, and 72°C 20 s. From 40 ~ 99°C, the temperature for the melting curve is increased by 2°C per second and fluorescence signals are collected. In order to reduce the errors, the quantitative RT-PCR is repeated once again. The average value is applied in the analysis. All samples were operated in duplicate and the mean value of each duplicate was used for all further calculations.

2.7. In Vitro Fertilization of Oocytes. After maturation, the oocytes were exposed to 0.1% hyaluronidase (H-3506, Sigma) to remove most of the cumulus cells. They were washed three times in the fertilization solution and then transferred at 20 to 30/drop into 50 µL fertilization droplets.

TABLE 2: Effect of BCB concentration on the selection and nuclear maturation after IVM of sheep oocytes (replicates = 3)^a, (mean ± S.D,%).

BCB concentration (μM)	N	BCB+, $n(\text{mean} \pm \text{S.D.}, \%)$	BCB+ oocytes in MII, $n(\text{mean} \pm \text{S.D.}, \%)$
26	342	228(66.15 ± 11.89)	197(86.16 ± 3.23)Aa
52	181	125(69.08 ± 0.21)	71(56.62 ± 2.43)Bb
Control	126		98(77.78 ± 2.75)c

^aValues in the same column with different small letters have differed significantly ($P < 0.05$); values with different large letters have extremely significant differences ($P < 0.01$).

We evaluated the motility of sperm cells under an inverted microscope and separated the motile sperm fraction by swim-up. The frozen semen was thawed in the test tube in a 39°C water bath. Then, 50 μL of the thawed semen was placed in two conical tubes under 0.5 mL fertilization medium (SOF [31] with 20% estrus sheep serum, 6 IU/mL heparin sodium, and 100 IU/mL gentamicin) and incubated for 20 min in a humidified 5% CO_2 atmosphere at 38.6°C. After incubation, 250 μL from the top of each tube was removed and pooled in a 1.5 mL centrifuge tube and centrifuged at 200 $\times g$ for 4 min. Viable spermatozoa collected at the bottom of the tube were washed with 1 mL fertilization medium and centrifuged again. Then, the sperm concentration was adjusted to 6×10^6 sperm/mL. Subsequently, 25 μL of sperm suspension was added into each fertilization droplet to obtain a final concentration of 2×10^6 sperm/mL. The dishes were incubated for 18 h at 38.6°C in a humidified 5% CO_2 atmosphere.

2.8. In Vitro Embryo Culture. After fertilization, the cumulus cells were removed from the presumptive zygotes using a fine pipette in culture medium (SOF [31] supplemented with 3 mg/mL BSA) and washed three times. Groups of 50 to 80 zygotes were transferred to 4-well plates containing 500 μL of culture medium at 38.6°C in a humidified atmosphere containing 5% CO_2 , 5% O_2 , and 90% N_2 .

2.9. Experimental Design

2.9.1. Experiment 1: Effect of BCB Concentration on Oocytes Selection and IVM. The objective was to determine the effect of varying concentrations of BCB on oocyte selection and embryonic development. Two concentrations (26 and 52 μM BCB in mPBS) were evaluated. The oocytes were exposed to the BCB for 90 min and then classified as BCB+ or BCB-. The percentages of the BCB- and BCB+ selected oocytes were recorded for each group, and then exposed to maturation medium. Control oocytes were washed and placed in the maturation medium immediately. After maturation for 24 h, the percentage of oocytes that reached the MII for each class was evaluated. The experiment was repeated three times. Based on the results of Experiment 1, 26 μM BCB was used in the succeeding experiments.

2.9.2. Experiment 2: Effects of BCB Screening on In Vitro Culture Efficiency. After classification, both BCB+ and BCB- oocytes were pooled and used for IVP of embryos; the maturation, cleavage, and blastocyst production rates were

recorded. After the selection, the oocytes were maintained in the maturation medium for 24 h. Some of the oocytes were removed randomly, blown in 0.1% (w/v) hyaluronidase to remove all the cumulus cells. Oocytes with polar bodies were recorded as mature oocytes, some oocytes from the pool were in MI or GV stage, so they were without polar body. The rest of the oocytes underwent IVF-IVC, after fertilization for 48 h. Cleavage rate (cleavage/oocyte) was recorded. Oocytes without cleavage were discarded. The day of fertilization was recorded as Day 0. At Day 7, the blastocyst efficiency (number of blastocysts/oocyte number) was recorded. The experiment was repeated three times.

2.9.3. Experiment 3: Oocyte Diameters Selected by BCB Test.

The oocytes from the maturation efficiency in Experiment 2 were selected randomly for diameter measurement. NIS-Elements, the image analysis software, and the NICON microscope camera system were used to measure the diameter of BCB+ and BCB- oocytes, including the zona pellucida.

2.9.4. Experiment 4: GSH Content in Oocytes after BCB Selection.

BCB+ and BCB- oocytes were blown into 0.1% (w/v) hyaluronidase (Sigma) to remove all cumulus cells. The samples were prepared using a GSH kit (China, Biyuntian) according to the manufacturer's instructions. The GSH content of the BCB+ and BCB- oocytes was measured using a microplate reader.

2.9.5. Experiment 5: Variations in Gene Expressions of GDF9, Mater, and Zar1 in Oocytes after the BCB Test.

The cumulus cells were removed from the IVM-oocytes. Real-time polymerase chain reaction probes were applied to test the gene expression of *GDF9*, *mater*, and *zar1*.

2.10. Statistical Analysis. The data from Experiment 1 were analyzed by one-way ANOVA. One-sample *t*-test was applied to the remaining experimental data. The software used was SPSS 11.0. At $P < 0.05$, the difference between treatments was considered significant. At $P < 0.01$, the difference was considered extremely significant. Data are expressed as mean \pm standard deviation.

3. Results

3.1. Experiment 1: Effect of BCB Concentration on Oocytes Selection and IVM. Table 2 shows the percentage of the selected oocytes and the rates of nuclear maturation obtained after exposure to 26 and 52 μM BCB. The percentages of

TABLE 3: Comparison of developmental efficiency of BCB selected oocytes after the *in vitro* fertilization (repeated 3 times)^a.

Oocyte classification	N	Oocytes in MII	Cleavage ^b	Blastocyst ^b
BCB+	201	172(86.16 ± 3.23)A	170(84.31 ± 2.13)A	53(34.4 ± 13.55)A
BCB-	91	46(50.94 ± 1.63)B	43(36.19 ± 3.67)B	3(6.73 ± 6.85)B

^aValues in the same column with different large letters have extremely significant differences ($P < 0.01$).

^bPercentage calculated from total number of oocytes.

TABLE 4: Diameter of BCB+ and BCB- oocytes selected by BCB test (replicates = 3)^a.

Oocyte classification	N	Diameter (μm)
BCB+	44	163.37 ± 4.50a
BCB-	44	159.25 ± 7.75b

^aValues in the same column with different small letters have differ significantly ($P < 0.05$).

TABLE 5: GSH content in BCB+ oocytes and BCB- oocytes (replicates = 3)^a.

Oocyte classification	N	GSH Content (pM)
BCB+	90	6.39 ± 1.19A
BCB-	90	0.26 ± 0.11B

^aValues in the same column with different large letters have extremely significant differences ($P < 0.01$).

BCB+ oocytes obtained after staining with 26 μM (66.15%) and 52 μM BCB (69.08%) showed no significant difference; however, the maturation rate of 26 μM BCB+ oocytes (86.16%) was significantly higher ($P < 0.01$) than those in the 52 μM BCB (56.62%), and higher ($P < 0.05$) than those in the control group (77.78%).

3.2. Experiment 2: Effects of BCB Selecting on In Vitro Culture Efficiency. Table 3 shows the differences of BCB selected oocytes in terms of maturation rate, cleavage, or blastocyst production. The rate of maturation, cleavage, and blastocyst of BCB+ oocytes was 86.16%, 85.29%, and 34.4%, respectively, which are significantly higher than those of BCB- oocytes ($P < 0.01$) (50.94%, 36.19%, and 6.73%, resp.).

3.3. Experiment 3: Oocyte Diameters Selected by BCB Test. Table 4 shows the diameter of BCB+ and BCB- oocytes. The mean diameter of BCB+ oocytes was 163.37 ± 4.50 μm (mean ± standard deviation), significantly greater ($P < 0.05$) than the diameter of BCB- oocytes (159.25 ± 7.75 μm).

3.4. Experiment 4: GSH Content in Oocytes after the BCB Selection Test. Table 5 shows the GSH content of oocytes after the BCB selection test. The GSH content in BCB+ oocytes (6.39 pM) was extremely higher than that of BCB- oocytes (0.26 pM) ($P < 0.01$).

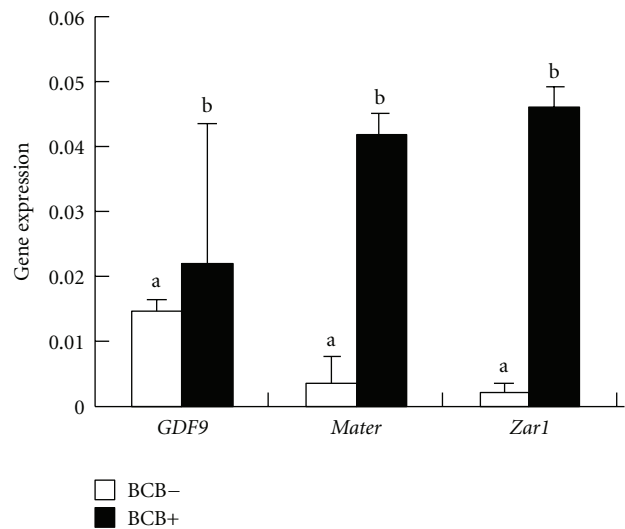


FIGURE 1: RNA expression of *GDF9*, *mater*, and *zar1* in oocytes after 24 h maturation. The black column represents RNA expression of *GDF9*, *mater*, and *zar1* in BCB+ oocytes. The white Column represents RNA expression of *GDF9*, *mater*, and *zar1* in BCB- oocytes.

3.5. Experiment 5: Variations in Gene Expressions of *GDF9*, *Mater*, and *Zar1* in Oocytes after the BCB Test. The expressions of the three maternal mRNA in BCB+ oocytes (*GDF9*, *mater*, and *zar1*) were significantly larger ($P < 0.05$) than in BCB- oocytes. The results are shown in Figure 1.

4. Discussion

In the study [7–17], immature oocytes stained by BCB. BCB+ oocytes showed higher rates of maturation, fertilization, and development to the blastocyst stage than BCB- oocytes. BCB can stain the cytoplasm and is an indicator of G6PDH activity. During the course of their growth, immature oocytes are known to synthesise a variety of proteins, including glucose-6-phosphate dehydrogenase (G6PDH). The activity of this protein is decreased once this phase has been completed and oocytes are then likely to have achieved developmental competence. In the growth and development of oocytes, G6PDH activity changes significantly with oocyte growth [4, 5, 10]. Thus, oocytes that have finished their growth phase show decreased G6PDH activity and exhibit cytoplasm with a blue colouration (BCB+), while growing

oocytes are expected to have a high level of active G6PDH, which results in colourless cytoplasm (BCB⁻).

Previous reports using the BCB test in pigs found that 13 μM BCB can predict the maturation rate of oocytes better than 26 μM [7, 8]. However, studies on the effects of BCB concentration on prepubertal goat [10–12], heifers [13], buffalo [16], and cattle [13–16] oocytes found that rate of maturation, cleavage, and development into blastocyst of oocytes selected using 26 μM BCB were higher than in oocytes selected with 13 μM , 39 μM , and 52 μM BCB. Therefore, for the BCB selection of oocytes in different animals, the appropriate concentration of BCB needs to be determined. In terms of classification, sheep are closely related to goats and cattle. Thus, 26 μM and 52 μM BCB were selected for present study, which provided similar experimental results with prepubertal goat, heifers, buffalo, and cattle oocytes. The research has found that in the selection of sheep oocytes, 26 μM and 52 μM BCB achieved no significant difference in staining effect. In contrast, the rate of maturation of 26 μM BCB⁺ oocytes (86.16%) was extremely and significantly higher than BCB⁺ selected by 52 μM (56.62%) and higher than the control oocytes (77.78%). The 26 μM concentration allow us to obtain a high rate of selected oocytes. The percentage of BCB⁺ oocytes (66.51%) obtained in the present study, employing 26 mM BCB, seemed those similar to reported in heifer (62% [32]; 66% [13]), cow (58% [14]) oocytes, but low than porcine oocytes (91% [7], and 81% [8]). The percentage of BCB⁺ oocytes reported in oocytes recovered from 2-month-old goats and 3–6-month-old sheep were 30.1% [33] and 19% [34], respectively. They also reported that the BCB staining was influenced by oocyte morphology, thus differences in morphological selection criteria might be associated with the differences observed in percentage of BCB-stained oocytes among laboratories. This implies that 26 μM BCB was more suitable than 52 μM concentration for selecting sheep oocytes. And we considered that 26 mM BCB could be used effectively in the study of embryo metabolism without being lethal as it was described by Tiffin et al. [35] in preattachment cattle embryos.

In the subsequent IVM, IVF, and IVC experiments, BCB⁺ oocytes had significantly higher cleavage and blastocyst rates (85.29% and 34.4%, resp.) than BCB⁻ oocytes (36.19% and 6.73%, resp.). Thus, BCB staining can be used to greatly improve the *in vitro* culture efficiency of sheep oocytes. Elucidating the mechanism for the differences in oocyte quality as determined by BCB is the main purpose of our next experiment.

Oocyte diameter is a determinant factor to complete meiosis and acquiring full competence for embryo development [21], and it is closely related to oocyte developmental competence [19, 20, 31]. In our work we found that BCB test was helpful in selecting larger oocytes with $163.37 \pm 4.5 \mu\text{m}$ diameter (BCB⁺) compared to those of $159.25 \pm 7.75 \mu\text{m}$ (BCB⁻). This confirmed previous reports in cattle (152.6 μm versus 147 μm) [14], goat (136.6 μm versus 125.5 μm) [11], pigs (113.08 μm versus 100.29 μm) [9], heifer (152.6 μm versus 147.0 μm) [13], and prepubertal sheep (123.66 μm versus 106.82 μm) [34] for BCB⁺ and BCB⁻, respectively.

After IVM, we found a significant increase of 12 μm of the internal zona diameter in BCB⁻ oocytes, while BCB⁺ oocytes maintained the same diameter after 24 hours of *in vitro* culture. Croet et al. [36] described the relationship of oocyte diameter to follicle size in adult goats, as well as to *in vitro* culture efficiency. When the follicle develops from 0.5 mm to 5 mm, the oocyte diameter grows from 96 μm to 146 μm , and the blastocyst efficiency of *in vitro* culture increases from 6% to 26%. Kim et al. [20] obtained the same result in similar studies involving pigs. Damiani et al. [37] found that if the oocyte volume is relatively small, its competence for protein synthesis, energy metabolism, and migration of cytoplasmic organelles are decreased significantly. So this finding confirms that BCB⁺ oocytes may have finished their growth phase when they are directly recovered from the ovary. This is the probable reason why oocytes of relatively small diameter have poorer viability.

Though the biochemical basis of BCB metabolism in oocyte is not fully understood, some evidence for the ability of BCB to play a role as electron acceptor and thereby become colourless during the electron flow induced by treated oocytes was then divided into BCB⁻ (colorless cytoplasm, increased G6PDH) and BCB⁺ (colored cytoplasm, low G6PDH) on their ability to metabolize the stain has been reported [14]. And we also observed that blastocyst development increased as the ovine oocyte diameter increased. Thus, the percentage of blastocysts was significantly higher in BCB⁺ oocytes than BCB⁻ oocytes.

Luberda [23] found that the maturation of oocyte cytoplasm involves a series of complex molecular reactions, including biological macromolecule synthesis, protein phosphorylation, and activation of certain metabolic pathways. The main function of GSH in oocytes is antioxidant, to prevent the oocyte from being poisoned by reactive oxygen species (ROS), while maintaining the meiotic spindle forms, and protecting the spindle from oxidative damages [38]. In addition, GSH concentration is very important for the maturation and fertilization of oocytes. In the maturation period of oocytes, GSH accumulation can improve the cytoplasmic maturation of oocytes and protect oocytes from oxidative damage in the embryonic developmental process after the fertilization [39, 40]. Thus, the GSH content can reflect the oocyte quality. This experiment examined GSH content in oocytes of different grades by BCB selection. The GSH content in BCB⁺ oocytes was 6.39 pM, significantly higher than 0.26 pM in BCB⁻ oocytes ($P < 0.01$). This indicates that BCB⁺ oocytes have better quality than BCB⁻ oocytes.

In the different developmental stages of oocytes and embryos, *GDF9*, *mater*, *zar1*, and other maternal genes were expressed differently [27–29]. In the study on the expression of three types of maternal mRNA (*Mater*, *Zar1*, and *GDF9*) in sheep oocytes and early embryos, our laboratory [30] found that with oocyte maturation and embryo development, the expression gradually declines. Thus, upon the maturation of oocytes, mRNA expression of the three maternal genes in completely matured oocytes is very low. After 24 hours of maturation, the mRNA expression of the 3 maternal genes in BCB⁺ oocytes was significantly lower than in the BCB⁻

oocytes, indicating that BCB+ oocytes have better quality than BCB− oocytes.

In conclusion, based on the comparison of *in vitro* culture, oocyte diameter, GSH content, maternal gene expression (*GDF9*, *mater*, *zar1*) between the BCB+ and BCB− sheep oocytes, BCB+ sheep oocytes were fully demonstrated to have better quality than BCB− sheep oocytes. Therefore, the BCB test is a useful method for selecting more competent immature sheep oocytes.

Authors' Contribution

L. Wang and J. Lin equally contributed in this paper.

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