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EFFECT OF BRILLIANT CRESYL BLUE ON IN VITRO MATURATION, FERTILIZATION, AND DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES

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ABSTRACT

Brilliant cresyl blue (BCB) staining is a non-invasive technique used to select developmentally competent oocytes for in vitro embryo production. This study investigated the developmental competence of bovine oocytes with blue cytoplasm (BCB+). Ovaries were collected from healthy cows within 30 minutes' post-slaughter. Cumulus-oocyte complexes (COCs) were aspirated from medium-sized ovarian follicles (4–8 mm), selected for quality, and incubated in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 26 µM BCB. Based on cytoplasmic staining, oocytes were classified as BCB+ (oocytes with blue cytoplasm) or BCB− (unstained cytoplasm). A third group served as an unstained control. All groups were matured in TCM-199 for 20–22 h at 38.5 °C under 5% CO_2 and 90% humidity. Following semen preparation, oocytes were co-incubated with spermatozoa for 4 hours and further cultured in TCM-199. Cleavage was assessed after 2 days, and embryo development was monitored every 48 hours for 7 days.

BCB+ exhibited significantly higher ($P < 0.05$) rates of cumulus expansion (91%), nuclear maturation (83%), and cleavage (56%) compared to BCB− and control groups. Blastocyst development was also significantly improved in BCB+ (27%) compared to BCB− (11%) and showed a non-significant increase over control (17%).

These findings confirm that BCB staining is an effective tool for selecting high-quality bovine oocytes for in vitro embryo production.

Keywords: Bovine, brilliant cresyl blue (BCB), in vitro maturation, fertilization, embryo development.

INTRODUCTION

Reproductive biotechnologies such as in vitro fertilization (IVF) and in vitro maturation (IVM) have revolutionized livestock production by enabling accelerated genetic improvement and the efficient propagation of high-value animals (Sirard, 2018). These techniques are widely used in cattle breeding programs, particularly in combination with embryo transfer, to maximize the reproductive potential of genetically superior females (Callesen, 2010; Fair, 2010; Lonergan and Fair, 2014). However, one of the major

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limitations of IVF efficiency remains the heterogeneous quality of oocytes collected from donor animals, especially those sourced from slaughterhouses or donors with unknown reproductive histories (Rizos *et al.,* 2002).

The developmental potential of oocytes is largely determined by their cytoplasmic and nuclear maturity, which are difficult to assess by morphology alone. Traditional morphological evaluation, while useful, has limited predictive power for determining oocyte competence (de Loos *et al.,* 1992; Lonergan *et al.,* 1996). To address this, several biochemical and molecular markers have been explored to better predict oocyte quality, including glucose-6-phosphate dehydrogenase (G6PDH) activity.

BCB staining is a non-invasive cytochemical method that exploits G6PDH activity as an indirect marker of oocyte maturity (Alm *et al.,* 2005; Ericsson *et al.,* 1993). G6PDH plays a central role in the pentose phosphate pathway and is highly expressed in metabolically active, growing oocytes. As oocytes complete their growth phase and acquire meiotic competence, G6PDH activity decreases. Therefore, oocytes that retain the blue stain (BCB+) are considered developmentally competent, while those that decolorize the dye (BCB−) are regarded as less mature and more metabolically active (Manjunatha *et al.,* 2007; Mertens *et al.*, 2001; Rodríguez-González *et al.,* 2002).

Numerous studies have reported that BCB+ exhibit significantly higher rates of nuclear maturation, fertilization, and blastocyst development compared to BCB− (Alm *et al.,* 2005; Ishizaki *et al.,* 2009; Pujol *et al.,* 2004). Moreover, BCB selection has been successfully applied across various species, highlighting its versatility as a tool for improving the efficiency of in vitro embryo production (Catalá *et al.,* 2011; Roca *et al.,* 1998).

The present study was designed to evaluate the effectiveness of BCB staining for selecting competent bovine oocytes based on G6PDH activity. Specifically, we aimed to assess the impact of BCB staining on in vitro maturation, fertilization, cleavage, and progression to the blastocyst stage, thereby validating its utility as a reliable selection criterion for bovine IVF protocols.

MATERIALS AND METHODS

This study was conducted in Animal Reproduction Research Institute – Giza, Egypt.

The chemicals in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA).

1. Recovery of immature oocytes

Ovaries were obtained from clinically normal cows of unknown age and reproductive status within 30 minutes after slaughter at EL-Bagor abattoir. They were transported in a thermos filled with warm saline solution (0.9% NaCl) containing gentamicin, and delivered to the lab within 1 to 2 h (El-Naby *et al.,* 2013).

Once in the lab, ovaries were rinsed with warm saline to eliminate blood and debris. Then ovaries were kept in a 37°C water bath during oocyte retrieval (Raghu *et al.,* 2002).

Using an 18-gauge needle attached to a 10 ml syringe, follicles ranging from 3–8 mm in diameter were aspirated. The contents were transferred into a 15 ml conical tube (Neglia *et al.,* 2003) and allowed to settle for 10–15 minutes. Approximately 5 ml of the sediment was collected and placed in sterile Petri dishes for further processing (Raghu *et al.,* 2002).

2. Preparation of the stain

A staining solution was prepared by dissolving 26 µM of Brilliant Cresyl Blue (BCB) in (DPBS) containing 0.4% BSA. The solution was sterilized through a 0.22 µm syringe filter and stored at 4°C (Pujol *et al.,* 2004). Cumulus-oocyte complexes (COCs) of good quality were incubated in this solution at 38.5°C in a 5% CO_2 atmosphere for 90 minutes. A control group remained in DPBS in the same conditions.

After incubation, oocytes were examined under stereomicroscope, and based on the cytoplasmic staining, they were categorized into BCB+ (blue cytoplasm, competent) and BCB− (colorless cytoplasm, less competent) as previously established by Alm *et al.* (2005); Rodríguez-González *et al.* (2002).

3. Oocytes Maturation

Post-staining, selected COCs were cultured in TCM-199 medium supplemented with Earle's salts, 10 µg/ml FSH, 10% FCS, 50 µg/ml sodium pyruvate, 2.6 mg/ml sodium bicarbonate, and 50 µg/ml gentamicin. Groups of 10–15 oocytes were placed in 100 µl droplets of medium covered with mineral oil, and incubated for 20–22 hours at 38.5°C in 5% CO_2 with 90% humidity. Maturation was evaluated by observing cumulus expansion and nuclear status using aceto-orcein staining under a phase contrast microscope (Nandi *et al.,* 1998).

4. Semen preparation and fertilization

Frozen bovine semen was sourced from the Animal Reproduction Research Institute and processed following the method described by (Neglia *et al.,* 2003).

Mature oocytes were washed three times in sperm-TALP and transferred into fertilization droplets (10 oocytes per drop). Co-incubation of gametes occurred for 4 hours at 38.5°C in 5% CO_2 and 90% humidity. Fertilization was assessed 24 hours post-insemination by identifying cleavage into 2- or 4-cell stages (Gasparrini *et al.,* 2008).

5. Developmental Stages

Presumptive zygotes (10 per 50 µl droplet) were cultured in TCM-199 at 38.5°C under 5% CO_2 with 90% humidity. The culture medium was replaced with fresh medium every 48 h (Badr, 2009).

Embryo development was monitored at 48-hour intervals for up to 7 days to assess morula and blastocyst development under a binocular microscope (Olympus, Japan).

6. Statistical Analysis

All experiments were performed in triplicate. Data were analyzed using one-way ANOVA with the UNIVARIATE procedure of SAS software (version 9.2, SAS Institute, Cary, NC, USA). When significant differences were detected, means were separated using Duncan's multiple range test (post hoc) at a significance level of $P < 0.05$. Results were expressed as mean ± standard error (SEM).

RESULTS

Data presented in (Table 1) demonstrated significant increase ($P<0.05$) in nuclear maturation, cumulus expansion, cleavage, and blastocyst development in BCB+ compared to both control and BCB−.

Table 1: Effect of BCB Staining on Bovine Oocyte Maturation and Embryo Development.

	No of oocyte	Control	BCB +	BCB -
Nuclear maturation	**100**	**65±4.79 ^{b}**	**83±3.775 ^{a}**	**52±5.02 ^{c}**
Cumulus expansion	**100**	**71±4.56 ^{b}**	**91±2.87 ^{a}**	**64±4.82 ^{b}**
Cleavage	**100**	**37±4.85 ^{b}**	**56±4.98 ^{a}**	**33±4.72 ^{b}**
Blastocyst development	**100**	**17±3.77 ^{ab}**	**27±4.46 ^{a}**	**11±3.14 ^{b}**

Values are presented as Mean ± SEM. Values with different superscript letters in the same row differ significantly ($P < 0.05$).

BCB+ showed a significantly ($P < 0.05$) higher cumulus expansion than BCB- and control ones (91±2.87, 64±4.82; 71±4.56, for BCB+, BCB- and control groups, relatively). At the same time, BCB+ revealed a significant ($P<0.05$) higher nuclear maturation than BCB- and control ones (83±3.775, 52±5.02; 65±4.79, for BCB+, BCB- and control groups, respectively). Additionally, BCB+ revealed a significantly ($P<0.05$) higher cleavage rate than BCB− and control groups (56±4.98, 33±4.72; 37±4.85, for BCB+, BCB- and control groups, respectively). On the other hand, BCB+ showed a significant ($P < 0.05$) increase than BCB^- and a non-significant increase, compared to the control ones (27±4.46, 11±3.14; 17±3.77, for BCB+, BCB- and control groups, correspondingly).

Representative microscopic images of BCB+ and BCB− oocytes are shown in (Figure1). The BCB+ oocytes demonstrated a distinct blue cytoplasm, confirming low G6PDH activity and higher competence, whereas BCB− oocytes appeared unstained, indicating higher G6PDH activity and lower developmental competence.

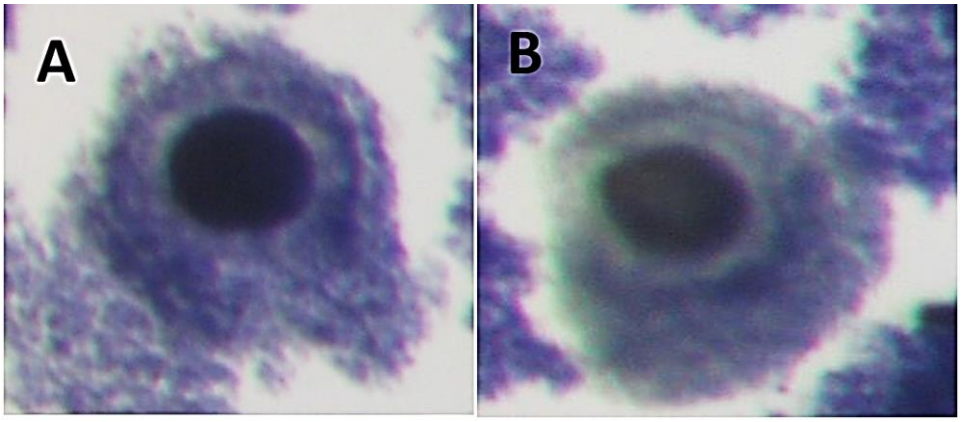

Figure 1: Representative images of bovine oocytes after BCB staining (original magnification approximately 400×). (A) BCB^+ oocyte showing a blue cytoplasm, (B) BCB^- oocyte with an unstained cytoplasm.

In addition, representative images of embryos at the morula and blastocyst stages produced from in vitro culture of selected oocytes are presented in (Figure 2). These images illustrate the successful progression of BCB+ oocytes to advanced embryonic stages.

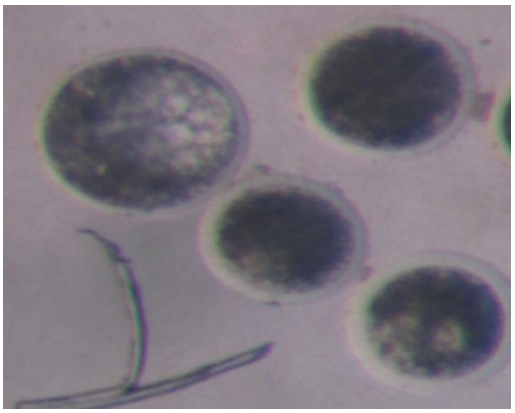

Figure 2: Representative images of bovine embryos at the morula and blastocyst stages derived from in vitro culture of oocytes selected by BCB staining (original magnification 100×)

DISCUSSION

Brilliant Cresyl Blue test, initially described by Alm *et al. (*2005) has proven to be a reliable, non-invasive technique for assessing oocyte developmental competence in bovine species. Our findings are consistent with previous research, demonstrating that BCB+ showed enhanced maturation, fertilization, and embryonic development, compared to BCB− and control groups. Multiple studies have confirmed that oocytes staining positively with BCB exhibit superior developmental potential and produce a higher number of blastocysts. This outcome is largely attributed to the selection of oocytes that are more mature and metabolically advanced. (Ishizaki *et al.,* 2009) reported similar observations, noting that BCB+ tended to be larger, with higher fertilization rates and a greater proportion of embryos progressing normally (Alm *et al.,* 2005; Manjunatha *et al.,* 2007; Rodríguez-González *et al.,* 2002). The significantly higher rates of nuclear maturation, cumulus expansion, cleavage, and blastocyst development in the BCB+ group indicates that BCB staining can reliably differentiate between metabolically competent and incompetent oocytes.

Including an unstained control group in this study ensured that any differences observed between BCB+ and BCB− oocytes were attributable to their developmental competence, and not to possible effects of BCB exposure itself. The control group allowed us to validate the non-invasive nature of BCB staining.

BCB^+ exhibited a blue cytoplasm, indicating low activity of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme highly active in growing (immature) oocytes (Alm *et al.,* 2005; Ericsson *et al.,* 1993).

The advantage of using BCB (Hillier, 2008) lies in its ability to distinguish oocytes that have completed their growth phase by identifying those with reduced glucose-6-phosphate dehydrogenase (G6PDH) activity, which is highly active in immature oocytes. BCB+ oocytes retain the blue stain, indicating their cytoplasmic maturity has been achieved, unlike BCB− oocytes that lack this staining due to ongoing metabolic activity.

This study used 26 µM BCB, a concentration previously applied in other species, such as goats (Rodríguez-González *et al.,* 2002), heifers (Pujol *et al.,* 2004) ; and cows (Alm *et al.,* 2005). The percentage of BCB+ obtained here (approximately 65%) aligns with reported values for heifers by (Pujol *et al.,* 2004) and buffalo by (Manjunatha *et al.,* 2007) and was lower than the seen value with porcine oocytes (Ericsson *et al.,* 1993; Roca *et al.,* 1998). The consistently higher nuclear maturation rates in BCB+ oocytes suggest that BCB staining does not interfere with maturation processes and may help identify oocytes with better developmental competence., similar to previous reports in goats (Rodríguez-González *et al.,* 2002), heifers (Pujol *et al.,* 2004); cows (Alm *et al.,* 2005), and buffalo (Manjunatha *et al.,* 2007).

Furthermore, reduced maturation in BCB− may be attributed to their incomplete growth (Alm *et al.,* 2005). Although the biochemical mechanism behind BCB's interaction with COCs is not fully understood, it is hypothesized that BCB acts as an electron acceptor and becomes colorless through G6PDH-mediated reactions, as reported by Alm *et al.* (2005). Thus, measuring G6PDH activity via BCB staining serves as an indirect indicator of oocyte viability.

CONCLUSION

This study demonstrated that brilliant cresyl blue (BCB) staining is an effective, non-invasive method for identifying developmentally competent immature bovine oocytes. BCB^+ exhibited

significantly higher rates of in vitro maturation, fertilization, and subsequent development compared to BCB^{-} and control groups. These findings confirm the utility of BCB staining as a valuable tool for improving the efficiency of in vitro embryo production in cattle.

Ethical approval

This study was conducted according to the standards of the Research Ethics Committee M /86 Faculty of Veterinary Medicine, Mansoura University, Egypt.

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تأثير صبغة زرقة الكيريزيل اللامعة على نضوج و إخصاب و نمو بويضات الأبقار

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تُعد صبغة زرقه الكيريزيل اللامعه (BCB) وسيلة غير جراحية لاختيار البويضات القادرة على التطور بشكل جيد لإنتاج الأجنة خارج الجسم الحي. هدفت هذه الدراسة إلى تقييم الكفاءة التنموية للبويضات الإيجابية للـ (BCB+) في الأبقار. تم جمع المبايض من أبقار سليمة بعد الذبح، واستخلاص البويضات غير الناضجة من الجريبات المتوسطة الحجم، ثم تصنيفها إلى ثلاث مجموعات: بويضات موجبة للصبغة (BCB+)، وسالبة للصبغة (BCB−)، ومجموعة ضابطة غير مصبوغة. تم تحضين جميع المجموعات في وسط TCM-199 للنضج لمدة ٢٠–٢٢ ساعة، ثم تم تخصيبها بالحيوانات المنوية المجمدة، ومتابعة تطورها إلى مراحل الانقسام والتكون الجنيني لمدة ٧ أيام.

أظهرت النتائج تفوقًا معنويًا للبويضات BCB^+ من حيث اتساع الخلايا التراكمية (٩١٪) والنضج النووي (٨٣٪) ونسبة الانقسام (٥٦٪) مقارنةً بالمجموعتين الضابطة و BCB^- كما سجلت مجموعة BCB^+ أعلى نسبة تطور إلى مرحلة الكيسة الأريمية (٢٧٪) مقارنةً ب (11%) BCB^-، مع فرق غير معنوي مقارنةً بالمجموعة الضابطة(17%).

تؤكد هذه النتائج فعالية صبغة BCB كأداة دقيقة وآمنة لاختيار البويضات الجيدة لتقنيات الإخصاب خارج الجسم الحي في الأبقار، مما يعزز من كفاءة إنتاج الأجنة ويُسهم في تحسين الأداء التناسلي.