In vitro maturation of goat oocytes selected using Brilliant cresyl blue staining

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Abstract

The present study was conducted to assess the developmental competence of goat oocytes selected using Brilliant cresyl blue (BCB) staining. Goat ovaries were collected from the slaughtered animals with unknown reproductive history. The oocytes retrieved by aspiration technique were selected based on morphology and subjected to BCB staining. Brilliant cresyl blue staining is based on the activity of glucose-6-phospahte dehydrogenase (G6PDH) enzyme synthesised by the oocytes. The cytoplasm remains blue in oocytes that have finished the growth phase (BCB+) while the growing oocytes remain colourless (BCB-). The stained and unstained oocytes were subjected to in vitro maturation separately to assess cumulus cell expansion index and polar body extrusion. A total of 206 culture grade oocytes were subjected to study, out of which, 76.75 ± 2.38 per cent of oocytes showed positive to BCB staining and 23.21 ± 2.38 per cent were negatively stained. Significantly higher maturation rate was observed in BCB+(92.89 ± 2.37%) oocytes than BCB-(29.72 ± 2.46%). The present study concluded that BCB staining can be used for selecting goat oocytes with good cytoplasmic maturation for further in vitro embryo production.

Key words: *Goat oocytes, in vitro maturation, Brilliant cresyl blue*

Running title: *In vitro* maturation of goat oocytes selected using Brilliant cresyl blue staining

Selection and identification of good quality oocytes is the critical step for *in vitro* embryo production which in turn ensures better *in vitro* maturation (IVM) and blastocyst formation (Egerszegi *et al*., 2010). Generally, the oocyte quality is determined by the morphological characters like number of cumulus cell layers and uniformity of the cytoplasm. A homogeneous population of oocytes with the same morphological characters differ in their developmental competence. Thus, BCB staining is proposed to assess the cytoplasmic competence which is predominantly responsible for oocyte competence.

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Brilliant cresyl blue is a vital stain that allows to determine the intra cellular activity of the glucose-6-phospahate dehydrogenase (G6PDH) which is an enzyme synthesised in the cytoplasm of growing oocytes but remains low in the oocytes that have finished their growth phase. As the enzyme is active during growth phase, immature oocytes reduce BCB to a colourless compound whereas, oocytes that have finished their growth phase show decreased activity of the G6PDH and their cytoplasm remain blue (Shabankareh *et al*., 2014). Studies in different farm species revealed that BCB selected oocytes were having uniform distribution of mitochondria, more lipid content and also high glutathione level all indicating good developmental competence (Ghoneimy *et al*., 2017). The purpose of this study was to use BCB staining as a selection tool to identify more competent goat oocytes having good cytoplasmic maturation for IVM and further processing for *in vitro* embryo production.

Goat ovaries transported to the laboratory within two hours of slaughter were washed several times in normal saline and removed excess tissue debris to reduce contamination. The visible surface follicles were aspirated and their contents were collected using a 10mL disposable syringe attached to 20G needle. Oocyte collection medium (OCM) was used for retrieving cumulus oocyte complexes (COCs). Preliminary selection criteria for COCs were based on morphological characteristics like uniformity of cytoplasm and compactness of cumulus cells (Shioya *et al*., 1988). The COCs were graded according to the number of cumulus layers present around the oocyte (Koeman *et al.,* 2003) as Grade-A ($\geq$3 cumulus layers), Grade-B (1-2 cumulus layers), Grade-C (denuded) and Grade-D (expanded cumulus). Grade A and B oocytes were selected for further *in vitro* studies.

The washed oocytes were subjected to BCB (B-5388-Sigma) staining by incubating at a concentration of 26µM at 37°C for 90 min. The stained oocytes were washed three times in maturation medium and grouped as BCB+ (blue coloured) and BCB- (colourless) depending on the blue colouration of cytoplasm (Fig.1). *In vitro* maturation was carried out separately for BCB+

and BCB- oocytes. Microdroplets of 100µL maturation medium were prepared and 20 to 25 oocytes were transferred to each microdroplet under mineral oil. Incubation was carried out at 38.5ºC in a humidified atmosphere under 5 per cent CO_2 for 27h.

After IVM, the maturation status of oocytes was assessed based on the degree of cumulus cell expansion and extrusion of first polar body in the perivitelline space. A total of 265 goat oocytes were collected from 353 follicles from 94 goat ovaries and classified based on the number of cumulus cell layer and uniformity of cytoplasm. Number for follicles obtained per ovary was 3.44 ± 0.26 and the number of COCs per ovary was 2.84 ± 0.19 with an oocyte recovery rate of 75.07 per cent. The maximum yield of COCs were obtained for grade A (48.43 ± 2.49%) followed by grade B (27.23 ± 2.22%). The yield of grade C and D were 12.28 ± 2.43 and 12.03 ± 2.46 per cent respectively. Since, only Grade A and B were considered as culture quality oocytes, they were selected for further *in vitro* studies. Out of 206 culture grade oocytes selected, 76.75 ± 2.38 per cent of oocytes showed positive to BCB staining and 29.72 ± 2.46 per cent oocytes were negative to BCB staining (Table 1). Maturation was assessed by cumulus cell expansion index following IVM. Most of the matured BCB+ oocytes were having a cumulus expansion index of grade one and two, indicating good quality and BCB- oocytes were having more per cent of unexpanded oocytes of grade zero, indicating poor quality oocytes. The overall maturation rates of BCB+ oocytes were found to be 92.89 ± 2.37 and 29.72 ± 2.46 per cent, respectively (Table 1). On statistical analysis, there was significant ($p<0.05$) differences between the maturation rate of BCB+ and BCB- oocytes.

**Table 1.** Yield and *in vitro* maturation rate of BCB selected goat oocytes

BCB Staining	Yield (%)	*In vitro* maturation rate (%)
BCB+	76.78 ± 2.38[a]	92.89 ± 2.37[a]
BCB-	23.21 ± 2.38[b]	29.72 ± 2.46[b]

** Significant at 0.01 level
Means having different letter as superscript differ significantly within a column

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Improvement in the oocyte screening can be achieved by the application of BCB staining in different species. The G6PDH activity was associated with oocyte diameter, lipid content, mitochondrial distribution and high glutathione content and those oocytes were having good cytoplasmic maturation and higher developmental competence (Wu *et al*., 2007). The percentage of BCB+ oocytes were higher than BCB- oocytes as described by several authors (Mohammadi-Sangchehmeh *et al*., 2014 and Abazari-kia *et al*., 2014). Usually, the oocytes were selected on the basis of morphological criteria alone, but oocytes that appear similar in morphology might vary in their developmental capabilities post insemination. Hence, application of an additional selection tool to choose oocytes with good cytoplasmic maturation, such as BCB staining would improve the IVEP outcome. In the present study, a higher yield of BCB+ oocytes than negative could be due to the fact that a preliminary selection was done based on morphology. Rodríguez-González *et al*., (2003) observed more maturation rate for BCB+ oocytes than BCB-. Cumulus expansion is the indicator of the ability to provide energy, transport of the messenger molecules or mediate the effects of hormone during the period of maturation and presence of cumulus investment increases the fertilisation and embryo development rate compared with that of denuded oocytes (Tange *et al*., 2004).

Good maturation rate indicates the quality oocytes and therefore good developmental competence for further *in vitro* developments. The study revealed that the goat oocytes selected by BCB staining (BCB+ oocytes) had a higher per cent of maturation rate when compared to BCB- oocytes.
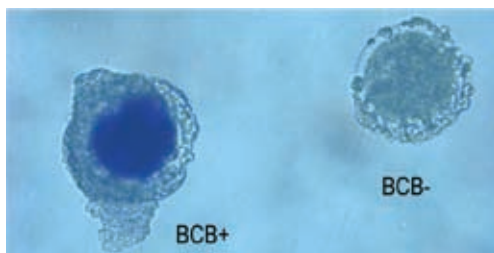
Fig. 1. Brilliant cresyl blue staining of goat oocytes (200X)

Summary

The oocytes selected by this method (BCB+) showed higher degree of cumulus cell expansion and maturation rate than BCB- oocytes. Brilliant cresyl blue being a non-invasive stain, the selected oocytes can further be used for *in vitro* fertilization and embryo production. Hence, this staining method can be used as an effective tool for the selection of good quality goat oocytes for *in vitro* embryo production.

Conflict of interest

The authors declare that they have no conflict of interest.

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