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Raghvendra Kumar
Animal Biotechnology Centre, ICAR-National Dairy Research Institute, Karnal, Haryana, India

Abhishek Thakur
Animal Biotechnology Centre, ICAR-National Dairy Research Institute, Karnal, Haryana, India

Corresponding Author:
Raghvendra Kumar
Animal Biotechnology Centre, ICAR-National Dairy Research Institute, Karnal, Haryana, India

Comparative properties of mammalian oocyte sorted through brilliant cresyl blue staining

Raghvendra Kumar and Abhishek Thakur

Abstract
Reproductive efficiency of oocyte plays pivotal role in female productivity. Oocyte quality and maturation level designate its developmental competence. Brilliant Cresyl blue (BCB) staining to immature oocytes is randomly used to select competent oocyte for *in vitro* embryo production (IVEP). BCB staining is based on the differential activity of glucose 6- phosphate dehydrogenase (G6PDH) in immature oocytes. BCB staining sorts these oocytes into growing (BCB-) and grown (BCB+), respectively. Since, after staining with BCB, oocytes remain in a condition to be used further, this method is employed seeking for optimal results in *in vitro* production, and also it is simple, quick and feasible protocol. However, it is a matter of debate that prolonged morphological oocyte selections with BCB staining have any detrimental effects or not. Although, optimal results have been reported while using BCB staining, contrary reports are also available. The aim of this review is to summarise the properties associated with BCB+ oocyte and BCB- oocyte in a comparative way. Here we have also discussed evidenced pros and cons while using BCB staining.

Keywords: BCB staining, oocyte, developmental competence, embryo production

Introduction
Screening oocytes containing high developmental capacity is a major step for successful embryo production (Egerszegi *et al.*, 2010) [9]. Several studies conducted oocyte selection through BCB staining for monitoring oocyte quality in relevance to developmental competence across many domestic mammals (Pig, Sheep, Goat, Cow, and Buffalo). Further, good quality oocytes link itself with optimal maturation rate *in vitro*. Intrinsic quality of oocyte and post fertilization culture system affects the developmental competence. From the earlier reports it is evident that BCB+ oocytes shows more competency, more blastocyst, selects larger oocytes, higher rate of *in vitro* maturation and fertilization. BCB+ oocytes get fertilized and develop to morula and blastocyst stage comparatively with higher rate than morphologically selected oocyte. BCB stain selects oocytes which contain more copies of mitochondria and activation of maturation promoting factor (MPF). Moreover BCB- oocytes shows reduced fertilization and lower development, hypothesized because of delayed mtDNA replication and associated nuclear regulation. In a study, Wongsrikeao *et al.*, 2006 [38] documented that BCB staining showed negative impact on cleavage and development in porcine embryo models. Opiela *et al.*, 2008 [20] reported no significant differences among BCB+ and control group in blastocyst formation. Further, in an investigation Pawlak *et al.*, 2013 [23] observed high incidence of apoptosis and diameter variation in BCB+ oocytes. Recently, in a study Ahmed *et al.*, 2020 [2] reported that preimplantation embryo development was not different for BCB+ and BCB- buffalo cumulus oocyte complex. Thus reports from these authors raise question regarding use of BCB staining before *in vitro* maturation (IVM). So considering the qualitative properties using BCB stain from different authors, the aim of this review is to summarise available facts concerned with oocyte quality.

BCB Staining
There are several markers which can reflect the developmental competence of an oocyte out of which G6PDH activity is a prominent one that can be identified using the BCB staining test (Wongsrikeao *et al.*, 2006; Kempisty *et al.*, 2011) [38, 11]. BCB is a dye which is blue in its natural form but becomes colourless when reduced. From the evidence emerged in recent few years, it has been shown that G6PDH-mediated reduction of BCB can be a useful tool to select good quality oocytes from a heterogeneous population.

Oocytes recovered from the ovaries of slaughtered animals are heterogeneous in terms of age, breed, health status, reproductive performance, origin from follicles at different stages of growth and atresia etc. Hence, they vary in their quality and developmental competence. BCB staining is easy to perform, non-invasive and harmless, and the oocytes selected through BCB staining constitute a more homogeneous population than that obtained through morphological criteria.

G6PDH is synthesized within the oocytes (Mangia and Epstein, 1975) [13], particularly in the first half of the S phase during the oocyte growth (Wassarman, 1988) [37]. G6PDH degrades BCB, to a colourless product. G6PDH is active in the growing oocyte but has decreased activity in oocytes that have finished their growth phase, and which are likely to have achieved developmental competence (Wassarman, 1988) [37]. Thus, oocytes that have completed their growth phase show decreased G6PDH and exhibit cytoplasm with blue coloration (BCB+), whereas growing oocytes, which are expected to have a high level of active G6PDH, remain colourless (BCB-) after staining. On the basis of the activity of G6PDH, the oocytes can be divided into two categories (Fig.1) after being stained with BCB, BCB+ (oocytes with blue cytoplasm) and BCB- (unstained cytoplasm). The underlying molecular mechanism targeted by the BCB test, however, remains unclear.

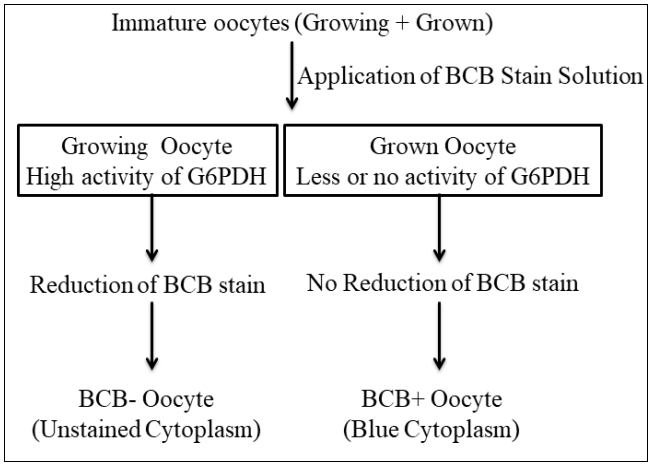

Fig 1: Hypothesized working of BCB staining to immature oocytes

Use of BCB concentration

Application of BCB staining to heterogeneous group of oocytes in different species reported to have variations in the ratio of BCB+ to BCB- oocytes. Several studies have reported that the rate of maturation, cleavage and blastocyst development of selected oocytes using 26 µM BCB was higher than that of 13 µM, 39 µM and 52 µM BCB selected oocytes in different species. These studies has been carried out in buffalo (Manjunatha *et al.*, 2007) [14], goat (Abazari-Kia *et al.*, 2014) [1], cattle (Alm *et al.*, 2005; Su *et al.*, 2012) [3, 33], mouse (Wu *et al.*, 2007) [39], and sheep (Wang *et al.*, 2012; Mohammadi-Sangcheshmeh *et al.*, 2014) [17, 36]. Thus the response of different concentrations of BCB seems to be species-specific. It is important to explore different levels of BCB concentration and oocyte selection incubation period (mostly 90 min.) in species-specific way. Further, an optimised species specific concentration of BCB should be taken for assessment of qualitative properties associated with mammalian oocyte.

Comparative properties associated with BCB+ and BCB- oocyte

Since the oocytes can be further processed after BCB staining, there have been several attempts to verify whether this technique can be used as a non-invasive, indirect indicator of the underlying characteristics of oocyte quality and developmental competence. BCB staining was used for the collection of oocytes with developmental competence to produce IVF embryos in mice (Wu *et al.*, 2007) [39] and other species of livestock, such as pig (Roca *et al.*, 1998) [26], sheep (Catala *et al.*, 2011; Mohammadi-Sangcheshmeh *et al.*, 2012) [8, 16], goat (Rodriguez-Gonzalez *et al.*, 2002) [27], cattle (Pujol *et al.*, 2004; Alm *et al.*, 2005; Silva *et al.*, 2013; Mirshamsi *et al.*, 2013) [25, 3, 32, 15] and buffalo (Manjunatha *et al.*, 2007) [14].

BCB+ oocytes have been shown to have a larger diameter, higher degree of maturation and higher fertilisation rate in prepubertal goats than BCB- oocytes (Rodriguez-Gonzalez *et al.*, 2002) [27]. Similarly, BCB+ oocytes had a higher diameter and higher percentage of oocytes hitting the blastocyst stage after IVF in heifers compared to BCB-oocytes (Pujol *et al.*, 2004) [25]. BCB+ oocytes were reported to be larger, and had higher mitochondrial activity, greater adenosine triphosphate (ATP) content, MPF activity and embryo developmental competence compared to BCB- oocytes in prepubertal sheep (Catala *et al.*, 2011) [8]. These authors reported that compared to those derived from BCB- oocytes, the number of cells in blastocysts derived from BCB+ oocytes was higher. Mohammadi-Sangcheshmeh *et al.*, 2012 [16] reported a major difference in oocyte diameter between BCB+ and BCB- oocytes in sheep. The maturation and cleavage rate of the BCB+ and control groups was significantly higher than that of the BCB- group. The blastocyst rate was higher for BCB+ group than for the control group which, in turn, was higher than for the BCB- group. In sheep, BCB+ oocytes were reported to be of better quality than BCB- oocytes based on oocyte diameter (Wang *et al.*, 2012; Mohammadi-Sangcheshmeh *et al.*, 2012) [36, 16], glutathione (GSH) content and maternal gene expression (Wang *et al.*, 2012) [36], maturation rates (Mohammadi-Sangcheshmeh *et al.*, 2012) [16] and blastocyst formation rates (Wang *et al.*, 2012, Mohammadi-Sangcheshmeh *et al.*, 2012) [36, 16].

Rodriguez-Gonzalez *et al.*, 2002 [27] reported that integration of the BCB test and the addition of cysteamine in the protocol of *in vitro* embryo production from prepubertal goat oocytes improved the rate of embryo development. Roca *et al.*, 1998 [26] reported that while selecting immature pig oocyte after staining with 13 µM BCB, data of selection effectiveness and percentage of penetrated oocytes obtained were more consistent when BCB+ oocytes were used. Egerszegi *et al.*, 2010 [9] analysed the meiotic progression, mitochondrial characteristics and fertilization ability of oocytes with different G6PDH activities and concluded that all of the three parameters were superior in the BCB+ compared with BCB- porcine oocytes. Pujol *et al.* 2004 [25] showed that the percentage of BCB+ oocytes reaching the blastocyst stage was significantly higher than the BCB- and control (morphologically selected) heifer oocytes, but lower than those of cow oocytes and concluded that heifer oocytes selected by BCB test i.e., BCB+ are larger and more competent for *in vitro* embryo production than control heifer oocytes.

Alm *et al.*, 2005 [3] demonstrated that growing oocytes had higher level of active G6PDH, while mature oocytes had low

G6PDH. These authors determined G6PDH activity by measurement of NADP reduction induced by glucose-6-phosphate as substrate in the cytosol of control, BCB- and BCB+ bovine oocytes. G6PDH activity was higher in BCB- than in control and BCB+ oocytes. They also observed the rate of maturation to metaphase II and the blastocyst rate to higher for control and BCB+ oocytes than for BCB- oocytes. Mirshamsi *et al.,* 2013 [15] demonstrated that combination of oocyte and zygote selection by BCB test enhanced the efficiency of selecting for high quality embryos, compared to the single BCB test in bovine. Silva *et al.,* 2013 [32] reported that nuclear maturation as indicated by polar body identification and cytoplasmic maturation, as indicated by migration of the cortical granules to the periphery and mitochondrial redistribution was significantly higher in BCB+ oocytes than in BCB- oocytes. Also, the blastocyst rate was higher in BCB+ than for BCB- or control oocytes. Castaneda *et al.,* 2013 [7] working with bovine oocyte found BCB+ oocytes contains 26% more cytoplasmic lipid than BCB- oocyte. The author proposed that higher lipid content may be useful for gaining functional basis to attain developmental competence. Salviano *et al.,* 2016 [28] reported that bovine BCB- oocyte negatively impact the capacity of BCB+ oocytes to undergo *in vitro* maturation, fertilisation and embryonic development. Santos *et al.,* 2015 [29] in porcine oocytes reported BCB staining negatively affects mitochondrial functions from immature to mature stage but gets restored during early embryonic development. In a study, Muasa *et al.,* 2015 [19] found increased proportion of BCB positive oocytes in >6mm follicles (81.1%) comparison to that of 1-3mm (73.1%) and >3-6 mm (76.5%) follicles. BCB positive oocytes recovered from 1-3 mm follicles had higher blastocyst rate (18.94%) than BCB negative oocytes (9.7%). These authors reported no difference in cleavage and blastocyst rates between BCB positive and negative oocytes for follicle with diameter of >3-6 mm. These researchers in Boran cows showed that rate of cleavage and blastocysts with BCB positive oocytes increases with the increase in follicle diameter. Thus follicular diameter may also play important function with associated gradient of activities of G6PDH.

Manjunatha *et al.,* 2007 [14] reported that the nuclear maturation rate was higher in BCB+ than in BCB- oocytes (86.2% vs 59.2%). The BCB+ oocytes yielded more blastocysts than BCB- oocytes suggesting that staining of buffalo oocytes with BCB before IVM is very effective in selection of developmentally competent oocytes for increased embryo production following IVF. In a study, Bhardwaj *et al.,* 2016[5] reported that the mean oocyte diameter was larger for the BCB+ than that of BCB- buffalo oocytes group (145.7 µm vs. 132.7 µm) and that the cleavage rate, blastocyst rate, and total cell number was higher in BCB+ than BCB- oocytes.

Despite availability of several reports confirming the efficacy of BCB staining for selection of developmentally competent oocytes for IVF in many species, there are only three reports available on the use of BCB staining of cytoplasm for selection of competent oocytes for somatic cell nuclear transfer (SCNT). Bhojwani *et al.,* 2007 [6] reported that selection of bovine COCs after BCB staining before IVM led to higher blastocyst rate after SCNT. BCB+ oocytes yielded a significantly higher blastocyst rate (39% vs 21%) than the control or BCB- oocytes (4%). Subsequently, Su *et al.,* 2012 [33] also reported that BCB+ oocytes yielded a significantly higher SCNT blastocyst rate and full term development rate of bovine SCNT embryos than the BCB- and control oocytes. Furthermore, BCB+ embryos generated more total cells, trophectoderm (TE) cells, and inner cell mass (ICM) cells, and fewer apoptotic cells than BCB- embryos. For selecting competent oocytes for Handmade cloning (HMC), Mohapatra *et al.,* 2015 [18] used BCB staining. These authors found that, the percentage of oocytes with clearly visible protrusion cone was substantially higher and the blastocyst production was nearly 2-folds more for BCB+ than BCB- oocytes. Blastocysts produced from BCB+ oocytes were also closer to those produced by IVF, as shown by similar apoptotic index, ICM cell number, ICM / TE ratio, H3K18ac global level, and BCL-XL and GATA2 expression levels, which were significantly different from those produced from BCB- oocytes.

Table 1: Summary of few properties associated with BCB+ and BCB- oocytes

S. No.	Properties	BCB+Oocytes	BCB- Oocytes	Donor Animal	Reference
1.	ATP Content	More	Less	Sheep	Catala *et al.,* 2011 [8]
2.	Blastocyst Rate	High	Low	Buffalo	Mohapatra *et al.,* 2015 [18]
3.	Cleavage Rate	High	Low	Buffalo	Bhardwaj *et al.,* 2016 [5]
4.	Diameter	More	Less	Buffalo	Bhardwaj *et al.,* 2016 [5]
5.	Glutathione Level	High	Low	Sheep	Wang *et al.,* 2012 [36]
6.	Lipid Content	High	Low	Bovine	Castaneda *et al.,* 2013 [7]
7.	Mitochondrial Activity	High	Low	Sheep	Catala *et al.,* 2011 [8]
8.	MPF	High	Low	Sheep	Catala *et al.,* 2011 [8]
9.	MtDNA copy number	High	Low	Pig	Tsai *et al.,* 2016 [35]
10.	No. of cell in blastocyst	High	Low	Bovine	Su *et al.,* 2012 [33]
11.	Nuclear Maturation	High	Low	Buffalo	Manjunatha *et al.,* 2007 [14]
12.	Cytoplasmic Maturation	High	Low	Cattle	Silva *et al.,* 2013[32]
13.	Mitochondrial Redistribution	High	Low	Cattle	Silva *et al.,* 2013 [32]
14.	Death Rate	Lower	Higher	Pig	Liu *et al.,* 2018 [12]

Genetic merits among BCB+ and BCB- oocytes

Torner *et al.,* 2008 [34] using a microarray platform reported that between BCB+ and BCB- bovine oocytes, several developmentally significant genes were found to be differentially expressed. It has been shown in porcine models that in comparison with control oocytes, BCB+ oocytes contained more transcripts of genes controlling sperm-oocyte interaction (Antosik *et al.,* 2009; Kempisty *et al.,* 2011) [4, 11]. Liu *et al.,* 2018 [12], identified 155 genes that were substantially differentiated between BCB negative and BCB positive oocytes through single-cell transcriptome sequencing on porcine germinal vesicle (GV) stage oocytes. These authors reported genes such as *cdc5l*, *ldha*, *spata22*, *rgs2*, *paip1*, *wee1b*, and *hsp27*, which were enriched in functionally important signaling pathways like Oocyte meiosis, spliceosome development, nucleotide excision repair,

including cell cycle control. Mohapatra *et al.,* 2015[18] reported that blastocysts formed from BCB+ oocytes and those derived from IVF, showed similarity in the global level of H3K18ac, the apoptotic index and expression level of the anti-apoptotic gene BCL-XL, which was greater than blastocysts developed from BCB- oocytes. OCT4 and SOX2 expression levels were higher and GATA2 levels were lower in BCB+ than in BCB- blastocysts, while DNMT1, DNMT3a, NANOG and CDX2 did not differ significantly among the two groups. The study inferred, since BCB+ blastocysts had better developmental abilities and found close to IVF blastocysts than BCB- blastocysts, BCB staining may prove to be an effective method for selection of high quality oocytes for Hand-made cloning. These comparative studies suggests that high throughput transcriptomics and proteomics on control, BCB- and BCB+ oocytes can bring us new candidate marker related to oocyte developmental competence.

Impact on oocyte while using BCB stain

Wongsrikeao *et al.,* 2006[38] concluded that BCB staining had a detrimental effect on cleavage and growth of porcine embryos after *in vitro* maturation. Staining COCs recovered from adult goats with BCB does not increase the efficacy of the collection of competent oocytes for IVF (Katska-Ksiazkiewicz *et al.,* 2007) [10]. As the Bax transcript level in BCB-oocytes was significantly higher compared to non-stained oocytes, Opiela *et al.,* 2008[20] suggested that oocytes subjected to BCB staining had a propensity to apoptosis. Opiela *et al.,* 2010 [21] did not notice major variations in blastocyst rate between BCB+ and control oocytes. Pawlak *et al.,* 2011 [22] found a higher incidence of chromosomal defects in porcine oocytes when stained with BCB. Compared to BCB- equivalents, BCB+ oocytes of all the organisms that have been investigated are characterised by superior consistency. Due to the relatively high similarity in selected parameters that characterise cytoplasmic maturation of BCB+ and control oocytes, the findings of pawlak *et al.,* 2013 [23] do not support the application of BCB staining in a routine IVM procedure. Pereira *et al.,* 2014 [24] showed in equine model investigation that BCB staining might not be useful for the selection of competent oocytes before IVM. Shabankareh *et al.,* 2014 [31] while working on bovine oocytes measured developmental competence based on follicle size and selection using BCB stain. These authors reported that each BCB+ oocyte could not contribute to perfect embryo development. These authors also proposed that for the identification of oocytes competent for *in vitro* embryo development, the BCB test is not sufficient. In both BCB+ and BCB- oocytes, Scholkamy *et al.,* 2015 [30] reported higher DNA damage compared to non-stained control vitrified oocytes. These authors therefore concluded that immature oocytes subjected to brilliant cresyl blue staining have a propensity to damage DNA. Ahmed *et al.,* 2020 [2] found no variations between BCB+ and BCB-COCs in embryo development; therefore, oocyte selection based on BCB staining is not an efficient method for selecting competent buffalo COCs for development.

Conclusion

High yield with high quality of embryos are important feature for which *in vitro* embryo production systems are constantly being optimised. As oocyte quality determines the developmental competence and quality of the embryos developed *in vitro*, parameters that allow good quality oocytes to be separated from a pool of oocytes may be used for their selection. BCB staining selects oocytes with higher rates of cleavage and blastocyst formation in both IVF and SCNT. Oocyte diameter, MPF, ATP content, mtDNA replication and glutathione level are major determining factors in completing meiosis and acquiring complete embryo development. BCB selects oocyte with larger diameter and higher abundance of these determining factors and thus enhances the embryo production efficiency. However consideration of these properties may have variation along with species in use and concentration of BCB opted for staining. Some reports where blastocyst rate were not significantly different among BCB+ and control group, raises question for the use of BCB stain. Prolonged morphological selection may lead to detrimental effects on oocyte behaviour and compromise in its intrinsic quality. Additional time and cost requirements for preparation of oocyte for IVEP also considered as disadvantage in using BCB staining. Overcoming all these drawbacks, most of the recent reports suggest use of BCB stain before IVM as it selects superior quality oocytes and enhances efficiency of *in vitro* embryo production.

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