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Effectiveness of Using Brilliant Cresyl Blue Staining for Quality Evaluation and Developmental Competence of Immature and Mature Buffalo Oocytes

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ABSTRACT

The present study aimed to evaluate the effect of brilliant cresyl blue (BCB) stain on quality and developmental competence of mature and immature buffalo oocytes. Oocytes were exposed to BCB stain diluted in mDPBS (DPBS with 0.4% BSA) for 60 min at 38.5°C in a humidified air atmosphere before, after maturation. The cleavage and blastocyst rates were determined. Results showed the percentage of BCB^+: BCB^- in immature and mature buffalo oocytes was 54:45 and 70:30 respectively. In immature oocytes percentage of embryo cleavage rate was highly significantly (P<0.0001) increased in BCB^+ than BCB^- oocytes. Lower cleavage rates were reported in immature BCB^+ (P<0.05) and BCB^-(P<0.0001) compared to control non exposed oocytes. There was no significant difference between control and BCB^+ oocytes in cleavage rate but there was a significant (P< 0.05) decrease in mature BCB^- than control oocytes. In conclusion, BCB had a detrimental effect on immature buffalo oocytes, but had no effect on mature oocytes. Thus, it was recommended that BCB was not suitable for oocytes selection as it toxic, time and money consuming.
Keywords: Buffalo, oocytes, BCB, embryo development.

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INTRODUCTION

The in vitro production inefficiency of embryos has been attributed to oocyte quality at the start of maturation (Gasparrini, 2002). Quality of oocytes that developed into viable embryos was still an issue of major concern in assisted reproduction technologies (Ruvolo *et al*., 2013). It was known that good quality oocytes were associated with better maturation rate in vitro. Many reports used brilliant cresyl blue staining as a vital stain to identify the quality oocytes in several species such as mice (Wu *et al*., 2007; Salimi *et al*., 2014), pig (Spikings *et al*., 2007), prepubertal goat (Rodriguez-Gonzalez *et al*., 2002; Abazari-Kia *et al*., 2014), buffaloes (Manjunatha *et al*., 2007), dog (Rodrigues *et al*., 2009), sheep

(Wang *et al*., 2012) and cattle (Pujol *et al*., 2004; Bhojwani *et al*., 2007; Salviano *et al*., 2015). But, the application of this test for a routine embryo production still remains an open issue.

Ericsson *et al*., (1993) suggested a simple test for the selection of porcine oocytes which had increased developmental competence; this test relied on measurement of glucose-6-phosphate (G6PDH) enzyme activity. Immature oocytes synthesized a variety of proteins, including glucose-6-phosphate dehydrogenase during their growth course (Mangia and Epstein, 1975). A function of G6PDH enzyme was to produce ribose sugars for nucleic acids synthesis through the pentose shunt (Alm *et al*., 2005).

BCB test involved staining immature cumulus oocyte complex (COCs) with BCB. The G6PDH enzyme converted the dye into a colorless form. Oocytes which stained blue (BCB^+, low G6PDH activity) were characterized by higher developmental competence (good quality), while the colorless oocytes (BCB^-, high activity of C6PDH) were characterized by low developmental competence (low quality) (Ericsson *et al*., 1993).

In animals, oocyte pre-selection with brilliant cresyl blue staining improves fertilization and blastocyst rates. Alm *et al*., (2005), Ishizika *et al*., (2009) reported that the use of BCB leaded to increase the efficiency of in vitro bovine blastocyst production from selected oocytes before maturation due to a significant higher rate of maturation to metaphase II for control and BCB^+ oocytes compared to BCB^- oocytes.

In spite of brilliant cresyl blue has been used to select competent oocytes in different species, this test has shown consistent results. Therefore, the present study was designed to investigate the effect of BCB on developmental competence of immature and mature oocytes as a method for quality evaluation.

MATERIALS AND METHODS

Chemicals

Chemicals for *in vitro* maturation including fetal calf serum and tissue culture medium (TCM 199) were obtained from Gibico BRL (Grand Island, New York, USA). Cysteamine, BCB and chemicals for in vitro fertilization were obtained from Sigma Chemical Company.

Oocyte Recovery

Buffalo ovaries were collected from abattoir within 2 h of slaughter. The ovaries were transported to the laboratory in physiological saline (0.9% NaCl) containing antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin) maintained at 30°C. Ovaries were washed three times in phosphate- buffered saline (PBS). Oocytes were aspirated from 2 to 5 mm follicles with a 20-gauge needle attached to a 5-ml syringe containing PBS with 0.3% bovine serum albumin (BSA), fraction V and antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin).

Brilliant Cresyl Blue Staining Test

Oocytes were washed three times in Dulbecco's PBS, modified by the addition of 0.4% BSA (mDPBS). The oocytes were exposed to 26 µM BCB diluted in mDPBS for 60 min at38.5 ºC in a humidified air atmosphere. The oocytes were then transferred to mDPBS, washed twice, and examined under a stereo zoom microscope. Oocytes with blue coloration of the cytoplasm were designated as BCB^+ and colorless were recorded as BCB^-.

In Vitro Oocyte Maturation

Oocyte maturation was carried out as previously described (Mahmoud, 2001). The selected oocytes were cultured in groups of 10 or 20 in 100 µl droplets of maturation medium. The medium consisted of TCM-199, 10% calf serum and 50 µg/ml gentamycin. The droplets were covered with mineral oil and were pre-incubated for a minimum of 2 h at 38.5°C 5% CO_2 in air with 95% humidity. Under this condition, the oocytes were added to droplets and incubated for 24h.

In Vitro Fertilization and Culture

Spermatozoa were treated as described by Niwa and Ohgoda (1988). Briefly, two straws of frozen buffalo semen were thawed in a water bath at 35–37°C for 1 min. The spermatozoa were washed twice by centrifugation (800×g for 10 min) in BO medium (Brackett and Oliphant, 1975) without BSA containing10 µg/ml heparin and 2.5 mM caffeine. The sperm pellets were diluted with BO medium containing 20 mg/ml bovine serum albumin to adjust the concentration of spermatozoa to 12.5×10^6 sperm/ml. Matured oocytes were washed three

times in BO medium containing 10 mg/ml BSA and were introduced into 100 µl droplets of sperm suspension (about 5–10 oocytes/droplet) under paraffin oil; the spermatozoa and oocytes were co-cultured for 5 h under the same culture conditions (5% CO_2, 38.5°C, 95% humidity). After that, the oocytes were washed in TCM-199 to remove attached spermatozoa. Groups of 10–20 oocytes were again replaced with previously prepared co-culture 100 µl droplet consisting of TCM-199+10% serum. Cleavage was assessed after 72 h of culture (day 0=day of insemination) and the number of embryos developing to the morula and blastocyst stages was assessed on days 5 and 7, respectively.

Statistical Analysis

Data were subjected to ANOVA using SPSS for Windows version 16.0, statistical software. Comparison of means of BCB^+, BCB^- and control was carried out by Duncan's Multiple Range Test. T-test was performed to compare between mature and immature exposed oocytes. Differences were considered to be significant at $P < 0.05$.

RESULTS

The mean proportion of immature and mature buffalo oocytes stained by 26 µM was given in Table (1) and Figure 1 (a & b). In immature oocytes, the percentage of stained BCB^+ oocytes were 54.8% and non-stained BCB^- oocytes were 45.2%. In mature oocytes, the BCB^+ oocytes were 70.4% and BCB^- were 29.6%. Thus, mature oocytes had high affinity for this stain than immature. There was significant difference (P<0.01) in mean percentage of staining between mature and immature oocytes in both BCB^+ and BCB^-.

The developmental competence of immature and mature oocytes after exposure to BCB was evaluated. About 268 immature, 281 mature and 144 control buffalo oocytes were exposed to 26µM of BCB stain (Table 2). In immature oocytes, the percentage of embryo cleavage rate was highly significantly (P<0.0001) increased in BCB^+ than BCB^- oocytes. Lower cleavage rates were reported in immature BCB+ (P<0.05) and BCB^-(P<0.0001) compared to control non exposed oocytes. The percentage of blastocyst rate was significantly (P<0.0001) higher in BCB+ than BCB^-oocytes. Comparing the blastocyst rates of BCB+ and BCB^-oocytes with control, there were significant differences (P<0.01) between BCB^- and control oocytes while there were no significant differences in BCB+ and control oocytes.

In mature oocytes, there was no significant difference between control and BCB^+ oocytes in cleavage rate but there was a significant (P<0.05) decrease in mature BCB^- than control oocytes. There were no significant differences among control, BCB^+ and BCB^- mature oocytes in blastocyst rates.

Comparing the immature with mature oocytes exposed to BCB, there were no differences in cleavage and blastocyst rates in both immature and mature BCB^+ oocytes. While there were significant (P<0.0001) increase in cleavage and blastocyst rates between immature and mature BCB^- oocytes.

DISCUSSION

The identification and selection of oocytes with good developmental competence was crucial for the successful embryo technologies. The present work studied the effectiveness of brilliant cresyl blue stain for oocytes selection before and after IVM on the cleavage and blastocyst rate in buffalo. The percentage of BCB+: BCB^- in immature and mature buffalo oocytes was 54:45 and 70:30 respectively. The result of immature oocytes was in agreement with (Manjunatha *et al*., 2007; Heleil and Fayed, 2010) in buffalo oocytes. While, there was no previous date about the percentage of staining mature oocytes. In our study, mature oocytes had high affinity for this stain than immature oocytes, this was attributed to that G6PDH enzyme was synthesized in oocytes during oogenesis and follicle growth, but this enzyme was inactive in oocytes that finished their growth phase. Therefore, when oocytes completed their growth phase, G6PDH activity was too small to reduce the staining so had blue coloration of cytoplasm (BCB^+) (grown) oocytes, while the growing oocytes remain colorless (BCB^-) (Knobil and Neill, 1988). So the lower G6PDH activity could be used as an ideal marker for induction of cytoplasmic maturation and to obtain the best maturation rates (Mohammadi-Sangcheshmeh, 2012).

In the current work, the effect of BCB stain on cleavage and blastocyst rate of immature

buffalo oocytes was studied. The percentage of stained BCB^+ oocytes were 54.8% and non-stained BCB^- oocytes were 45.2%., and percentage of embryo cleavage rate was highly significantly (P<0.0001) increased in BCB^+ than BCB^- oocytes. Lower cleavage rates were reported in immature BCB^+ (P<0.05) and BCB^- (P<0.0001) compared to control non exposed oocytes. So this stain had detrimental effect on immature oocytes and it was not recommended to apply as a routine procedure for oocytes selection. Our data was in agreement with Bols *et al*., (2010); Pawlak *et al*., (2014); Pereira *et al*., (2014) who stated that there was similarity in cytoplasmic maturation and fertilization outcome between positive BCB and control bovine, porcine, equine oocytes respectively, these authors didn't support the application of BCB staining in a routine IVM protocol. Moreover, Opiela *et al*., (2008) mentioned that the staining bovine oocytes had no significant effect in blastocyst rates, but had detrimental effect on blastocyst quality, and these oocytes showed a tendency toward apoptosis. Wongsrikeao *et al*., (2006); Kempisty *et al*., (2011) and Shabankareh *et al*., (2014) determined the toxicity of BCB staining by estimating the potential for fertilization and embryonic development of porcine oocytes subjected to double (before, after IVM) BCB staining, where double staining was detrimental for oocytes. Recently, Scholkamy *et al*., (2015) evaluated the utility of brilliant cresyl blue in selection of mature buffalo oocytes by using comet assay and found that there was a significantly higher DNA damage in both BCB^+ (P<0.05) and BCB^- (P<0.001) oocytes, compared to non-stained control vitrified oocytes.

Table 1: Immature and mature buffalo oocytes stained by 26 µM of brilliant cresyl blue (Mean ± S.E).

Type of oocytes	Total oocytes No.	Stained oocytes (BCB^+) No. (%)	Non-stained oocytes (BCB^-) No. (%)
Immature	288	160 (54.8±1.1)	128 (45.2±1.1)*
Mature	356	256 (70.4±3.3)*	100 (29.6±3.3)

* = P< 0.01(t-Test).

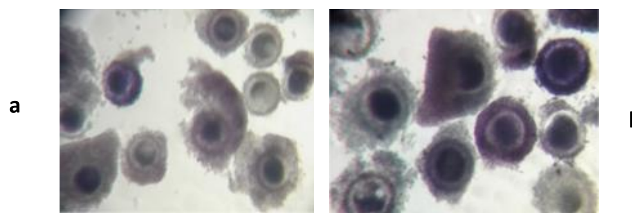

Figure 1: a, Immature and b, mature buffalo oocytes stained by BCB, blue oocytes indicate BCB^+

Table 2: Embryo development rate of immature and mature buffalo oocytes stained by 26 µM of BCB (Mean ± S.E)

Oocyte classification	Total inseminated oocytes	Cleavage No. (%)	Blastocyst No. (%)*
Immature	(268)		
BCB+	155	97 (62.8±3.1)^b	14 (9.1±0.9)^b
BCB^-	113	47(41.6±2.2)^a	4(3.7±0.4)^a
Mature	(281)		
BCB+	177	121 (67.8±3.5)^{bc}	23(11.8±1.6)^b
BCB^-	104	67 (63.6±1.5)^{bc}	9 (9.1±1.3)^b
Control	144	103 (70.4±1.5)^c	14 (10.7±2.2)^b

*Percent from total inseminated oocytes
Values within same column without common superscripts differ (P<0.05- P<0.01- P<0.0001).

Our result was in contrast to Manjunatha *et al*., (2007); Heleil and Fayed (2010), who mentioned that the maturation, cleavage and blastocyst rate was significant higher for BCB^+ than control, BCB^- buffalo oocytes. Also, Alm *et al*., (2005), Ishizika *et al*., (2009) reported that the use of BCB leaded to increase the efficiency of in vitro bovine blastocyst production from selected oocytes before maturation due to a significant higher rate of maturation to metaphase II for control and BCB^+ oocytes compared to BCB^- oocytes. Moreover, Catala *et*

al., (2011); Mohammadi-Sangchesmeh *et al*., (2012) reported that the higher mitochondrial activity and more active maturation-promoting factor at the metaphase II stage in BCB^+ as compared to BCB^- sheep oocytes.

To our knowledge, this work was the first investigation to study the relationship between stain exposure and mature oocytes. There was no significant difference between control and BCB+ oocytes in cleavage rate but there was a significant ($P< 0.05$) decrease in mature BCB^- than control oocytes. There were no significant differences among control, BCB^+ and BCB^- mature oocytes in blastocyst rates. These result might be due to the activity of glucose-6-phosphate dehydrogenase (G6PDH) declined in mature oocytes so this stain couldn't change the stain color by reduction (Abazari-Kia *et al*., 2014). There was an association between G6PDH activity with intracellular glutathione (GSH) content and meiotic competence. GSH was the major non protein sulphydryl compound in mammalian oocytes and gametes. GSH played critical roles in oocyte function including spindle maintenance and provision of reducing power needed to initiate sperm chromatin decondensation. Also, GSH concentration was higher in mature than immature hamster oocytes and declined after fertilization (Zuelke *et al*., 2003). Moreover, it accumulated to improve cytoplasmic maturation of oocytes and protect oocytes from oxidative damage in embryonic developmental process after fertilization (Ozawa *et al*., 2010; Wang *et al*., 2012).

On the other side, there were no differences in cleavage and blastocyst rates in both immature and mature BCB^+ oocytes. While there were significant increase in cleavage and blastocyst rates between mature and immature BCB^- oocytes. Also, there were significant differences between control, immature BCB^-, BCB^+ and mature BCB^- in cleavage rate. Thus, BCB stain had detrimental effect on both mature and immature oocytes but this effect was more obvious in immature oocytes, this was attributed to the sensitivity of immature oocytes to this stain. On the other side, the glutathione was synthesized during oocyte maturation and increased in mature oocytes (Zuelke *et al*., 2003), and had a profound impact on fertilization and embryo development (Eppig *et al*., 1996). Glutathione stabilized mitotic spindle against oxidizing agents in oocyte and was involved in enhancement of metaphase II, normal formation of egg and inhibition of two cell stage arrests (Nasr-Esfahani and Johnson, 1992).

CONCLUSION

In conclusion, there were no differences in blastocyst rates between BCB^+ and control immature and mature oocytes. So it was recommended that BCB is not suitable for oocytes selection as it toxic, time and money consume.

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