IN VITRO DIFFERENTIATION OF DEVELOPMENTALLY COMPETENT OOCYTES BY MONITORING GLUCOSE 6 PHOSPHATE DEHYDROGENASE (G6PDH) ACTIVITY WITH BRILLIANT CRESYL BLUE STAIN IN JAMUNAPARI GOAT

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ABSTRACT

A large proportion of oocytes recovered from abattoir mammalian ovaries fail to fertilize *in vitro* due to developmental incompetence. Detection of Glucose- 6-phosphate dehydrogenase (G6PDH) enzyme in the growing oocyte by brilliant cresyl blue (BCB) stain has emerged as an effective non-invasive alternate to determine the developmental competence of the oocyte over conventional methods. The present study elucidates the use of BCB test for detection of developmentally competent oocytes for *in vitro* embryo production in Jamunapari goat. To our knowledge, there is no report on this aspect. This study is based on the cumulative oocyte complexes (COCs) recovered from ovaries of 487 Jamunapari goats comprising 330 adult (≥ 1 year) and 157 young (< 1 year) goats collected from a Delhi abattoir. The COCs of 230 adults and 107 young goats were stained with BCB for 90 minutes before placing for maturation, while the rest served as control. The COCs displayed cleavage after 48 hours of co-incubation with sperm. The proportion of cleaved embryos from BCB^+ adult goats (81.78±1.88%) was significantly ($P \leq 0.05$) higher than BCB^-, but did not differ from the control ($P \geq 0.05$). There was no difference ($P \geq 0.05$) between the groups in young goats, and between the adult and young ($P \geq 0.05$). The embryos developed to blastocyst stage seven days post-fertilization, but the pace of development of COCs to reach blastocyst stage was faster in BCB^+ group than the other two (Control and BCB^-) groups. The proportion of blastocysts out of total cleaved embryos from BCB^+ adult (44.55±0.67%) and young (43.62±1.10%) goats were significantly ($P \leq 0.05$) higher than the BCB^- and control. There was no difference between the adult and the young ($P \geq 0.05$). The proportions of hatched blastocysts in BCB^+ adult (33.83±3.72%) and young (29.52±5.79%) goats were significantly ($P \leq 0.05$) higher than the control. There was no hatched blastocyst in BCB^- group. The blastocysts from all the three groups both in adult and young goats showed normal morphology. The average numbers of nuclei in the blastocysts recovered on the 7th day of culture in adult and young goats were 98.13±2.80 and 99.33±5.05, respectively. The differences between the groups and between the ages were non-significant ($P \geq 0.05$). This study implied that BCB stain could be used as a reliable marker for selection of developmentally competent oocytes for *in vitro* fertilization in adult as well as juvenile goats with equal precision.

KEY WORDS

Blastocyst, Brilliant Cresyl Blue, *In vitro* embryo production, Jamunapari goat

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INTRODUCTION

Slaughterhouse derived ovary is the common source of mammalian oocytes. The oocytes recovered from ovary are a heterogeneous population of both grown and growing oocytes. Their differentiation is crucial for *in vitro* fertilization, since fertilizing sperm recognizes only fully matured oocytes (Plachot and Mandelbaum, 1990). It has been observed that more than half (53.5%) of the bovine oocytes fail to fertilize (Rizos *et al*., 2005) due to developmental incompetence, which is the prime reason for setback in *in vitro* embryo production.

Immature oocytes secrete an enzyme, known as glucose-6-phosphate dehydrogenase (G6PDH), which is at a high level in immature oocyte (Mangia and Epstein, 1975), but drastically reduced upon attaining maturity (Tian *et al*., 1998). G6PDH is degradable by brilliant cresyl blue (BCB). The cytoplasm of mature oocytes stained with BCB gets blue colouration due to low level of G6PDH, while growing oocytes turn colourless because of high level of G6PDH. This has been successfully tested in different mammalian species that include cattle (Alm *et al*., 2005, Bhojwani *et a*l., 2007), buffalo (Manjunatha *et al*., 2007), goat (Rodrýguez-Gonzalez *et al*., 2002), sheep (Asghari *et al*., 2010) and pig (Wongsrikeao *et al*., 2006).

To our knowledge, there are no reports on the application of brilliant cresyl blue (BCB) test for selection of developmentally competent oocytes in Indian goats. The present study was undertaken to improve *in vitro* goat blastocyst production in Jamunapari goat, the best and the most grandiose dairy goat breed in India, with protracted age at maturity (Singh and Roy, 2003) that overstretches generation interval, and facing extinction due to declining number in its home turf (Rout *et al*., 2012).

MATERIALS AND METHODS

This study was conducted in the Animal Biotechnology Centre of the National Dairy Research Institute, Karnal, Haryana, India, during 2009.

Collection of oocytes: The ovaries of 487 Jamunapari goats comprising 330 adult (≥ 1 year) and 157 young (< 1 year) goats, ascertained on the basis of dental archetype, were collected from Delhi abattoir, and were transported to the institute laboratory in thermo flask containing 0.9% sterile normal saline fortified with antibiotics (50 µg / ml gentamicin sulphate) maintaing 32-35°C temperature within 3-4 h of collection. The ovaries were excised with the help of a sterile scissors and washed 4-5 times in 0.9% normal saline supplemented with antibiotics. Each ovary was placed in a Petri dish and covered with aspiration medium consisting of TCM-199 (HEPES modified) and bovine serum albumin (0.3% v / w). The follicles present on the surface of the ovary were punctured with the aid of an 18 gauge sterile needle and searched under stereo zoom microscope under 20 X magnification. Cumulus oocyte complexes (COCs) having more than 3 layers of cumulus cells and homogeneous ooplasm were selected for culture.

Treatment of oocytes with BCB: The selected COCs (230 from adult goats and 107 from young goats) were stained with brilliant cresyl blue (BCB) stain, while the rest (100 COCs from adult goats and 50 COCs from young goats) were maintained as controls. The COCs were washed three times in Dulbecco's PBS, modified with an addition of 0.4% BSA (mDPBS), and were exposed to 26 μM of brilliant cresyl blue (BCB) stain diluted in mDPBS for 90 minutes at 38.5°C in humidified air. The COCs were stained with BCB, and were transferred to mDPBS and washed twice, after which, they were examined under a stereomicroscope for the colour of the cytoplasm, and were graded to BCB+ (Blue colour) and BCB – (Colourless) oocytes. The oocytes with any degree of blue coloration to the cytoplasm (BCB+) were considered to be mature and the oocytes without blue cytoplasm (BCB-) were considered immature.

***In vitro* maturation of oocytes:** The COCs were washed 5-6 times in maturation medium consisting of TCM 199 (HEPES modified), 10 μg/ml LH, 5 μg/ml FSH, 1 μg/ml estradiol-17β, 0.05 mg/ml sodium pyruvate, 5.5 mg/ml glucose, 0.0035 mg/ml L-glutamine, 0.05 mg/ml gentamicin, 3 mg/ml BSA and 10% EGS (heat inactivated goat serum). The COCs (15-20 oocytes) were placed in 100 μl of droplet of maturation medium in a 35 mm Petri dish, covered with paraffin oil, and incubated in a CO_2 incubator (5% CO_2 in air) with maximum humidity at 38.5°C temperature for 24 hours.

Collection and capacitation of spermatozoa: Fresh semen was collected from a proven buck by using artificial vagina. Raw semen (50 μl) was placed in 2 ml sp-TALP medium (Parrish *et al*., 1998) in a tube and incubated for 15 minutes at 38.5°C temperature under 5% CO_2 in air. After incubation, the top 1.5 ml liquid was taken and washed two times in sp-TALP medium at 296 gravity (x g) for 7 minutes each to remove the seminal plasma. Finally, sperm pellet was suspended in 2 ml of fert-TALP medium, containing 50 g/ml heparin (Ktska-Ksiazkiewicz *et al*., 2004) and incubated at 38.5°C under 5% CO_2 in air at 38.5°C temperature for 1.5 h for capacitation.

***In vitro* fertilization:** Oocytes with expanded cumulous cells were taken out from maturation medium drops after 24 h of maturation. The expanded cumulus cells were then removed by repeated gentle pipetting in fert-TALP medium using sterilized Pasteur pipette. About 10-12 denuded oocytes were placed in 50 μl droplet of fert-TALP and covered with mineral oil and incubated at 38.5°C under 5% CO_2 in air for the equilibration of medium prior to *in vitro* fertilization. Capacitated sperm suspension of 50 μl ($2x10^6$ to $4x10^6$sperm/ml) was added to each 50 μl drop of fert-TALP medium containing denuded oocytes and co-incubated for 10 h at 38.5°C and 5% CO_2 in air with maximum humidity.

Culture of presumptive embryos: At the end of 10 h of co-incubation, oocytes were

taken out from fertilization drops and washed 5-6 times carefully in the embryo development medium (EDM) containing TCM 199 (HEPES modification), 30 µg/ml sodium pyruvate, 100 µg/ml L-glutamine, 50 µg/ml gentamicin, 10 µl/ml essential amino acids, 5 µl/ml non-essential amino acids, 10 mg/ml BSA (Fraction-V) and 10% FCS to remove attached spermatozoa without damaging the oocytes by gentle pipetting. Then the washed oocytes (10-15 in number) were put in 100 µl droplet of EDM in a 35 mm petridish and covered with mineral oil. This Petri dish was incubated in 5% CO_2 in air at 38.5°C and oocytes were examined for cleavage after 35 hours.

Blastocyst evaluation: The blastocysts were evaluated by staining them with Hoechst 33342 stain and cell numbers were counted under fluorescence inverted microscope (Nikon, Japan).

Statistical analysis: The data were analyzed by standard statistical procedure (Snedecor and Cochran, 1989).

Table-1. *In vitro* cleavage and blastocyst development rate of selected COCs based on BCB staining in adult and young goats (Based on four trials).

Attribute / Age	Control	BCB $^+$	BCB $^-$
	Cleavage % of total COCs		
Adult (≥ 1 year)	79.58±2.37^a (80) {80/100}	81.78±1.88^a (123) {123/150}	45.17±0.55^b (36) {36/80}
Young (< 1 year)	71.67±2.55^a (37) {37/50}	73.33±3.85^a (39) {39/53}	61.43±4.43^a (33) {33/54}
Adult vs. Young	NS	NS	NS
	Blastocyst % of cleaved embryos		
Adult (≥ 1 year)	22.69±1.67^a (18) {18/80}	44.55±0.67^b (55) {55/123}	5.05±2.94^c (2) {2/36}
Young (< 1 year)	22.42±2.42^a (8) {8/37}	43.62±1.10^b (17) {17/39}	2.56±2.56^c (1) {1/33}
Adult vs. Young	NS	NS	NS
	Hatched blastocysts % of total blastocysts		
Adult (≥ 1 year)	22.92±2.08^a (4) {4/18}	33.83±3.72^a (18) {18/55}	0.00±0.00^a (0) {0/2}
Young (< 1 year)	11.11±11.11^a (1) {1/8}	29.52±5.79^a (5) {5/17}	0.00±0.00^a (0) {0/1}
Adult vs. Young	NS	NS	—-

Note: (1) The figures are presented as Mean±SEM (Number). (2) The group means were compared by chi-square test. (3) The means with different superscripts in a row differed significantly (P≤0.05). (4) The figures in {parentheses} indicate the number of cleaved embryos/ blastocysts/ hatched blastocysts over the total number of COCs/ total cleaved embryos/total blastocysts, respectively. (5) NS= Non-significant at P≤0.05.

Table-2: Number of nuclei in blastocysts recovered on day 7 of culture.

Attribute / Age	Control	BCB $^+$	BCB $^-$	Pooled
Adult (≥ 1 year)	97.80± 3.11^a (5)	99.80± 7.16^a (5)	96.80 ± 3.11^a (5)	98.13± 2.80(15)
Young (< 1 year)	100.80± 9.68^a (5)	101.20± 10.57^a (5)	96.00 ± 4.95^a (5)	99.33 ± 5.05 (15)
Adult vs. Young	NS	NS	NS	NS

Note: (1) The figures are presented as Mean±SD (Number). (2) The group means were compared by t-test. (3) The means with similar superscripts in a row did not differ significantly (P≤0.05). (4) NS= Non-significant at P≤0.05.

RESULTS

The proportion (%) of the cleaved embryos out of total COCs, the proportion (%) of blastocysts out of the cleaved embryos, and the proportion (%) of hatched blastocysts out of total blastocysts in adults and young are presented in Table-1.

In this study, the COCs displayed cleavage (Figure-1) after 48 hours of co-incubation with sperm. The percentage of cleaved embryos out of total COCs, in the control, BCB+, and BCB- goats were 79.58±2.37%, 81.78±1.88%, and 45.17±0.55 % respectively in adults, and 71.67±2.55%, 73.33±3.85%, and 61.43±4.43% respectively in young goats. The percentage of cleaved embryos in BCB+ group was significantly ($P \leq 0.05$) higher than BCB- in adult goats, but did not differ ($P \geq 0.05$) from the control. The differences between the groups were non-significant ($P \geq 0.05$) in young goats. There was no difference ($P \geq 0.05$) between the adult and young in all the three groups (Control, BCB+, BCB-).

The embryos developed to blastocyst stage (Figure-2) seven days post-fertilization. The percentage of blastocysts out of total cleaved embryos, in the control, BCB+, and BCB- goats were 22.69±1.67%, 44.55±0.67%, and 5.05±2.94% respectively in adults, and 22.42±2.42%, 43.62±1.10%, and 2.56±2.56% respectively in young goats. The percentage of blastocysts was significantly ($P \leq 0.05$) higher in BCB+ goats than in the control and BCB-, both in adult and young goats. There was no difference ($P \geq 0.05$) between the adult and young in all the three groups (Control, BCB+, BCB-).

The percentage of hatched blastocysts (Figure-3) out of total blastocysts, in the control and BCB+ goats were 22.92±2.08% and 33.83±3.72%, respectively in adults, and 11.11±11.11% and 29.52±5.79%, respectively in young goats. There was no hatched blastocyst in BCB– group. The differences in the proportions of hatched blastocysts between the control and BCB+ goats were non-significant ($P \geq 0.05$), both in adult and young goats. There was no difference ($P \geq 0.05$) between the adult and young in both the groups (Control, BCB+).

Blastocysts from all the three groups (adults and young) showed normal morphology on 7^{th} day of culture, but COCs in BCB+ group reached blastocyst stage much faster than control and BCB- group. The number of nuclei in blastocysts was counted and no significant difference ($P \geq 0.05$) was found between different groups both in adult and young, and between the adult and the young goats (Table-2).

DISCUSSION

It is generally seen that a large proportion of goat oocytes fail to develop to blastocyst stage after maturation, fertilization and culture *in vitro*. While suboptimal culture conditions contribute to the poor development of embryos, the quality of oocytes is the main limiting factor (Alm *et al.*, 2005). Immature oocytes are generally arrested at the diplotene stage of meiosis-I. During the course of their growth, immature

oocytes synthesize a variety of proteins, including glucose-6-phosphate dehydrogenase (Mangia and Epstein, 1975). The activity of this protein is decreased once this phase has been completed and oocytes are then likely to have achieved developmental competence (Tian *et al*., 1998).

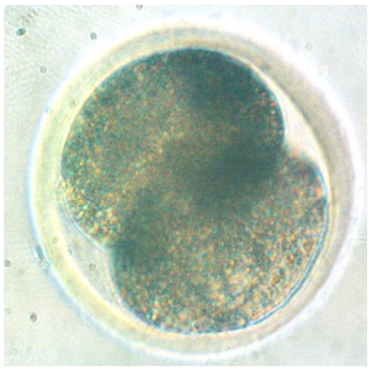
Brilliant Cresyl Blue (BCB) is a dye that can be degraded by G6PDH (Tian *et al*., 1998). Thus, the oocytes that have finished their growth phase show decreased G6PDH activity and exhibit cytoplasm with a blue coloration (BCB+), while growing oocytes are expected to have a high level of active G6PDH, which results in colorless cytoplasm (BCB-).

The method of COC recovery may influence the degree of heterogeneity of the recovered COCs. When ovaries are sliced, COCs may be recovered not only from antral follicles on the surface, but also from the smaller antral follicles from the inside of the ovary, which may be in earlier stages of follicular development after antrum formation (Alm *et al*., 2005). This influences the rate of blastocyst production.

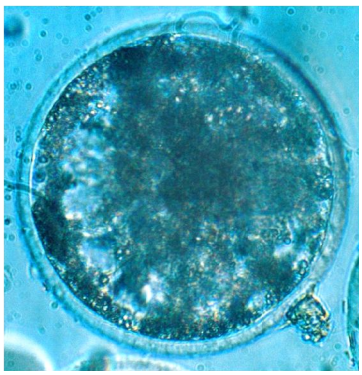
The proportion of BCB+ oocytes developed to blastocyst stage found in our study (44.55%) was higher than the report of Alm *et al*. (2005) in goats (34.1%) and Asghari *et al*. (2010) in sheep (34.5%) confirming successful use of BCB test in selecting fully mature oocytes in Jamunapari goat.

Jamunapari goat is the best and the most grandiose dairy goat breed in India. However, the protracted age at maturity of this breed (Singh and Roy, 2003) would reduce the magnitude of genetic improvement through selection because of prolonged generation interval.

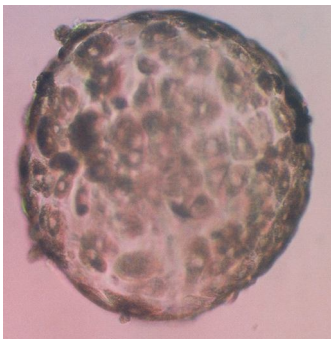
Our results showed that the use of juvenile animals for *in vitro* embryo production would reduce the generation interval, and thus improve the magnitude of genetic gain through selection without compromising blastocyst yield. This agreed with the findings of Rodriguez-Gonzalez *et al*. (2002). *In vitro* embryo production will also arrest the progress of inbreeding, and save the breed from genetic impoverishment caused by decline in genetic diversity of the breed in its home tract (Rout *et al*., 2012).



Fig, 1: Two cell stage goat embryo (400 X)



Fig, 2: Blastocyst stage goat embryo (400 X)



Fig, 3: Hatched blastocyst of goat (400 X)

CONCLUSION

The result of this study indicated that BCB stain can be successfully deployed to select mature oocytes, which would decisively enhance en-mass *in vitro* embryo production of Jamunapari goat, and save this precious germplasm from extinction.

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