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Developmental competence of Dromedary camel (*Camelus dromedarius*) oocytes selected using brilliant cresyl blue staining

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Summary

The objectives of the present studies were to investigate the developmental capacity of dromedary camel oocytes selected by brilliant cresyl blue (BCB) staining and to investigate the expression of select transcripts in germinal vesicle (GV) stage oocytes. These transcripts included BMP15 and GDF9 as important transcripts for folliculogenesis and oocyte development, Zar1 and Mater as maternal transcripts required for embryonic development, Cyclin B1 and CDK1 as cell cycle regulators and Oct4 and STAT3 as transcription factors. Dromedary camel oocytes were retrieved from ovaries collected at a local slaughterhouse. After exposure to BCB staining, cumulus–oocyte complexes (COCs) from BCB+, BCB− and control (selected based on morphological criteria) groups were subjected to *in vitro* maturation, *in vitro* fertilization and *in vitro* culture. For gene expression studies, after BCB staining cumulus cells were stripped off and the completely denuded GV stage oocytes were used for RT-PCR analysis of selected transcripts. BCB+ oocytes showed higher maturation, and fertilization rates compared with BCB− and control groups. Indices of early embryonic development, namely, cleavage at 48 hours post insemination (hpi), and development to morula at day 5 and day 7 blastocyst rates were also significantly higher in the BCB+ group. RT-PCR revealed a higher expression of BMP15, GDF9, Zar1, Mater, Cyclin B1, CDK1, OCT4 and STAT3 in good quality oocytes that stained positively for BCB (BCB+). Collectively, results provide novel information about the use of BCB screening for selecting good quality oocytes to improve *in vitro* embryo production in the dromedary camel.

Keywords: BCB, Brilliant cresyl blue, Dromedary camel, Oocytes, Gene expression

Introduction

The success of *in vitro* embryo production depends mainly on the selection of good quality oocytes (Ashry & Smith, 2015). Morphological evaluation of the cumulus–oocyte complex is a widely used method for selection of oocytes for *in vitro* embryo production, not only in the dromedary camel but in almost all other species. Brilliant cresyl blue (BCB) staining is a non-invasive method for screening for oocyte quality that has been used in different animal species (Roca *et al*., 1998; Rodriguez-Gonzalez *et al*., 2002; Bhojwani *et al*., 2007; Manjunatha *et al*., 2007; Wu *et al*., 2007a; Ishizaki *et al*., 2009; Rodrigues *et al*., 2009; Catalá *et al*., 2011; Wang *et al*., 2012; Silva *et al*., 2013; Pereira *et al*., 2014). Recent studies on human oocytes have also been reported (Alcoba *et al*., 2016, 2017). BCB staining depends on measuring glucose-6-phosphate dehydrogenase (G6PDH) activity. G6PDH is an enzyme synthesized by the growing oocytes (Mangia & Epstein, 1975; Pujol *et al*., 2004). G6PDH activity is reduced once the oocytes have finished the growth phase and possibly have acquired developmental competence (Wassarman, 1988; Tian *et al*., 1998). Therefore, oocytes that have finished their growth

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phase have low levels of G6PDH and exhibit a cytoplasm with a blue color (BCB+) while growing or less competent oocytes have a high levels of G6PDH, and are able to reduce the blue color of the dye, and display colorless cytoplasm (BCB−) (Ericsson *et al*., 1993; Tian *et al*., 1998). Previous studies in different animal species have demonstrated that screening of oocytes using BCB staining improved the development potential of the embryos derived from (BCB+) oocytes. Indices of early embryonic development, including cleavage, and development to morula and blastocyst, were significantly higher in BCB+ derived embryos (Alm *et al*., 2005; Bhojwani *et al*., 2007; Manjunatha *et al*., 2007; Wang *et al*., 2012). The molecular identity of oocytes screened with BCB staining is completely different in many aspects related to oocyte quality including gene expression (El Shourbagy *et al*., 2006).

Using BCB staining for screening camel oocytes has not been reported previously to the best of our knowledge. Therefore, the objectives of the current studies were to investigate the developmental competence of dromedary camel oocytes selected by BCB staining. Effects of BCB staining on oocyte maturation, fertilization and early embryonic development were elucidated. The differential expression of select candidate genes that are important for folliculogenesis and oocyte development [growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15)], plus cell cycle regulators [Cyclin B1 and cyclin-dependent kinase1 (CDK1)], maternal effect genes required for embryo development [zygote arrest factor 1 (Zar1) and Maternal Antigen that embryos require (Mater)] or genes that have transcription factor activity [OCT4 and Signal transducer and activator of transcription 3 (STAT3)], was also investigated.

Materials and Methods

Materials

Unless stated otherwise, chemicals and reagents were purchased from Sigma–Aldrich (S.A., Egypt).

Ovary collection and oocyte retrieval

Dromedary camel oocytes were retrieved from ovaries collected from local slaughterhouse (Cairo, Egypt). Oocytes retrieval and selection were performed as described elsewhere (Fathi *et al*., 2014). Briefly, the ovaries were transported to the laboratory within 2–3 h after slaughter in a thermos flask filled with prewarmed (30°C) sterile normal saline solution (NSS, 0.9% NaCl). Cumulus–oocyte complexes (COCs) were aspirated from 2 to 8 mm follicles using a 20-gauge needle attached to a 20-ml syringe (Moawad *et al*., 2011). Morphologically good quality oocytes were selected under a stereomicroscope.

Brilliant cresyl blue staining

BCB staining was carried out as previously described (Ashry *et al*., 2015). Briefly, BCB stain was diluted in Dulbecco's phosphate buffered saline containing 0.4% BSA (mDPBS). Morphologically good quality oocytes were stained with BCB at 38.5°C in a 5% CO_2 humidified air atmosphere. The control group was kept in mDPBS under the same conditions. After staining, COCs were washed twice with mDPBS and classified into BCB+, with a blue cytoplasm or BCB−, with a colorless cytoplasm. Firstly, we tested the effect of three different BCB concentrations (13, 26 and 39 µM) on oocyte maturation to select the appropriate staining protocol for camel oocytes. Based on the results of first experiment, 26 µM of BCB was used in the subsequent experiments.

In vitro embryo production

Dromedary camel oocyte recovery, *in vitro* maturation (IVM), *in vitro* fertilization IVF (IVF) and embryo culture were performed as described previously (Moawad *et al*., 2011; Fathi *et al*., 2014). Briefly, after BCB staining, selected COCs were matured in TCM-199 with Earle's salts (supplemented with 10 µg/ml FSH, 10% FCS, 50 µg/ml sodium pyruvate, 2.6 mg/ml sodium bi-carbonate, and 50 µg/ml gentamycin). COCs were cultured in 100-µl drops of maturation medium (10–15 oocytes/drop) covered with mineral oil at 39°C in 5% CO_2 in humidified air for 30 h. For assessment of nuclear maturation, cumulus cells were stripped off, then denuded oocytes were transferred to 1% sodium citrate solution for 3 min. Oocytes were fixed in ethanol:acetic acid (3:1) for 24 h, then stained with 1% orcein and examined under a phase contrast microscope in which MII stage oocytes were considered mature. *In vitro* fertilization was carried out using dromedary camel epididymal spermatozoa. Epididymal spermatozoa were collected by flushing the body and cauda epididymis and separated by swim up technique in the presence of 5 mM caffeine as a capacitating agent (Fathi *et al*., 2014). Matured COCs were co-incubated with spermatozoa (2×10^6 motile spermatozoa/ml) in 50-µl drops of fertilization medium (10 oocytes/group) at 39°C in 5% CO_2 humidified air for 18–20 h. After fertilization, cumulus cells were stripped by repeated pipetting. Fertilization was then determined by fixation and staining of presumptive zygotes using aceto-orcein staining as detailed above. Normal fertilization was evidenced by the presence of spermatozoon, sperm head, or male and/or female pronuclei in the ooplasm. The presence of more than one spermatozoon or

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Table 1 Primers used for RT-PCR analysis

Gene	GenBank accession number	Primer sequence
BMP15	XM_010998065.1	F: 5′–GCCACTACTTTGCCCCTGAT–3′; R: 5′–GGGGTGCAATGATCCAGTGA–3′
CDK1	XM_010985774.1	F: 5′–AAGTTAAGGGAGAGCGACCG–3′; R: 5′–AGCTCTGGCAAGGCCAAAA–3′
Cyclin B1	XM_010949351.1	F: 5′–TGTGTGCCCAAGAAGATGCT–3′; R: 5′–AGGGCGACCCAGACAAAAAT–3′
GAPDH	XM_010990867.1	F: 5′–GTCAAGGCTGAGAACGGGAA–3′; R: 5′–GTGTGTTGGGGGATCGAGTT–3′
GDF9	XM_010981399.1	F: 5′–CCATCAGTGGACCTGCTGTT–3′; R: 5′–CACTGAGGGGTCGAGCTTTT–3′
Mater	NM_001007814.2	F: 5′–GTTCCCCCAGAGCACAAAGA–3′; R: 5′–GATCTCCCGACAAGAGGCTG–3′
Oct4	NM_174580	F: 5′–GAAAGACGTGGTCCGAGTGT–3′; R: 5′–CAGGGAATGGGACCGAAGAG–3′
STAT3	XM_001494674	F: 5′–TCCCTGATTGTGACCGAGGA–3′; R: 5′–GTCCTTCTCCACCCAAGTGAA–3′
Zar1	XM_010992872.1	F: 5′–TGGGAAAGTGCCTACGTGTG–3′; R: 5′–AAGGAGAAGGGGCATGGAAA–3′

F: forward primer, R: reverse primer.

two pronuclei in the ooplasm was considered to demonstrate polyspermic fertilization.

Eighteen hours post insemination, presumptive zygotes were washed and cultured in modified KSOMaa (Khatir *et al.*, 2007), 5 zygotes/50 µl drop under mineral oil at 39°C in 5% CO_2 humidified air atmosphere until day 7 (day 0 = day of insemination). The number of cleaved embryos (2–8 cells) was determined at 48 hpi. The development to morula and blastocyst stages was evaluated at day 4 and day 7 post insemination respectively.

RNA isolation and real-time PCR

After exposure to BCB staining, cumulus cells were stripped off by repeated pipetting and GV stage oocytes from BCB+, BCB− and control groups (n = 20 oocytes/group, n = 4 replicates) were snap frozen for further use in RNA isolation. RNA extraction, cDNA synthesis and real-time PCR analysis of gene expression were carried out as described before (Bettegowda *et al.*, 2008; Ashry *et al.*, 2015). Briefly, an RNeasy® Micro Kit (Qiagen, Germantown, MD, USA) was used for total RNA extraction and cDNA synthesis was done using an iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) according to the manufacturer's protocols. Quantification of relative transcript abundance of each gene was done by the $2^{-\Delta\Delta Ct}$ method as described elsewhere (Livak & Schmittgen, 2001). Transcript abundance of the studied genes was normalized relative to the abundance of GAPDH as an endogenous control and expressed as fold change relative to the control group. PCR primers were designed using the NCBI Primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers sequences and GenBank accession numbers are presented in Table 1.

Statistical analysis

Differences in gene expression were analyzed by one-way analysis of variance (ANOVA) using SAS (SAS Institute Inc., NC, USA). For other experiments, percentage data were arcsine transformed before analysis with ANOVA. Differences amongst treatment means were detected using Fisher's protected least significant difference test (LSD).

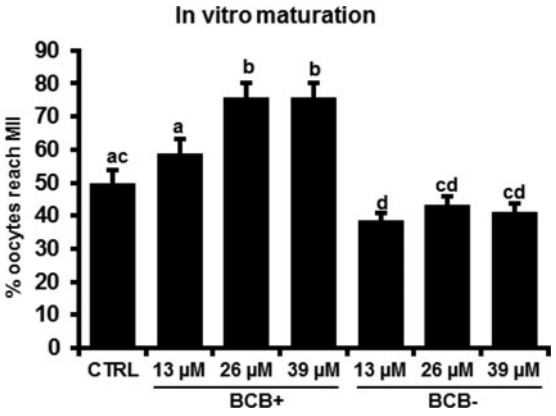

Figure 1 Effects of different BCB concentrations on camel oocyte maturation. Cumulus–oocyte complexes (COCs) from control, BCB+ and BCB− groups (n = 20–30 oocytes/group; n = 4 replicates) were subjected to *in vitro* maturation for 30 h, then nuclear maturation status was determined by phase contrast microscopy. Data are shown as mean ± standard error of the mean (SEM). ^{a,b,c,d}Values with different superscripts across treatments indicate significant differences ($P < 0.05$).

Results

Effect of BCB staining on oocyte selection efficiency and *in vitro* maturation

To determine the appropriate BCB concentration for staining of camel oocytes we tested three different BCB concentrations (13, 26 and 39 µM). As depicted in Fig. 1, staining of dromedary camel oocytes with 26 or 39 µM of BCB for 90 min resulted in a significant increase in the maturation rate of BCB+ oocytes

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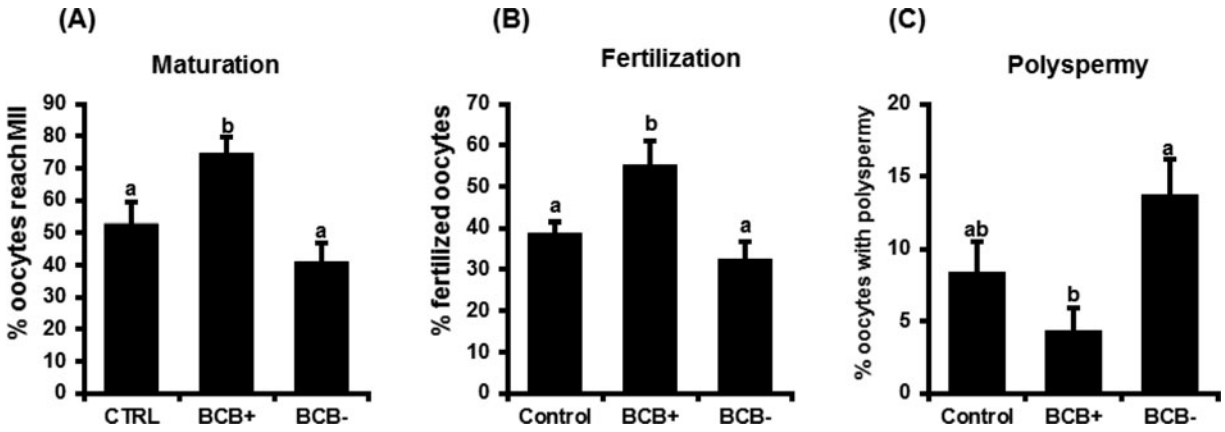

Figure 2 Effects of BCB staining on camel oocytes maturation and *in vitro* fertilization. After BCB staining, BCB+, BCB− and Control oocytes ($n = 4$ replicates, 20–25 oocytes/group) were subjected to *in vitro* maturation, and *in vitro* fertilization. The ratio of oocytes reaching MII at 30 h (*A*), normally fertilized oocytes (*B*) and polyspermic fertilization (*C*) were recorded. Data are shown as mean ± standard error of the mean (SEM). [a,b]Values with different superscripts across treatments indicate significant differences ($P < 0.05$).

compared with controls and the 13 µM BCB concentration. No significant difference in the maturation rate was observed between 26 and 39 µM concentrations. Based on the results of this experiment the 26 µM concentration was used for subsequent experiments.

Effects of BCB on *in vitro* maturation and fertilization of dromedary camel oocytes

In terms of *in vitro* maturation and fertilization, screening of camel oocytes with BCB staining before maturation resulted in significant increase in the maturation and fertilization rates and reduced the incidence of polyspermy in the BCB+ group. BCB+ oocytes showed a significantly higher maturation rate compared with BCB− and the control oocytes, with maturation rates of 74.48, 41, and 52.88% for BCB+, BCB− and control respectively (Fig 2*A*). *In vitro* fertilization rate was also increased in the BCB+ group with a low incidence of polyspermy (Fig. 2*B*, *C*). the fertilization rate was 55.33, 32.5 and 38.86%, while the polyspermy was 4.29, 8.4 and 13.75% for BCB+, BCB− and control groups respectively. Suggesting that screening of camel oocytes with BCB staining prior to *in vitro* maturation would select the more competent oocytes that have a higher potential to resume meiosis and able to be normally fertilized.

Effects of BCB staining on the development of early dromedary camel embryos *in vitro*

To further investigate the influence of BCB staining on camel oocyte developmental potential, *in vitro* produced presumptive zygotes of BCB+, BCB− and control groups were cultured *in vitro* up to day 7. Indices of early embryonic development; cleavage, development to morula and blastocyst stages were significantly improved in BCB+ groups compared with the BCB− and control groups. Cleavage rate at 48 hpi was 44.29, 22.5 and 32.84% for BCB+, BCB− and control groups respectively (Fig. 3*A*). the percentage of embryos that reached morula stage at day 5 was 29.8, 12.5 and 19.4% for BCB+, BCB− and control groups respectively (Fig. 3*B*), while blastocyst development rate at day 7 was 18.7, 3.75 and 9.77 for BCB+, BCB− and control groups respectively. Collectively, results suggested that screening of camel oocytes with BCB staining would select more competent oocytes for *in vitro* embryo production.

Expression of select candidate genes in dromedary camel oocytes screened with BCB staining

To investigate the effect of BCB staining on the molecular level, we analyzed the transcripts abundance of select transcripts in GV stage camel oocytes screened with BCB staining. Transcripts were selected based on the biological function in the oocytes and/or early embryos. Expression of BMP15 was >2-fold higher ($P < 0.05$) in BCB+ relative to BCB− and control groups, whereas no significant difference was observed between BCB− and control groups (Fig. 4*A*). Although, mRNA abundance for GDF9 was higher in the BCB+ group compared with BCB− oocytes, mRNA abundance did not differ between BCB+ and control groups (Fig. 4*A*, *B*). Transcript abundance of Zar1 and Mater was significantly higher ($P < 0.05$) in the BCB+ group versus BCB− and control groups. Both transcripts were significantly lower in the BCB− group relative to controls (Fig. 4*C*, *D*). Expression of cyclin B1 was >6-fold and 3-fold higher ($P < 0.05$) in the BCB+ group compared with the BCB− and

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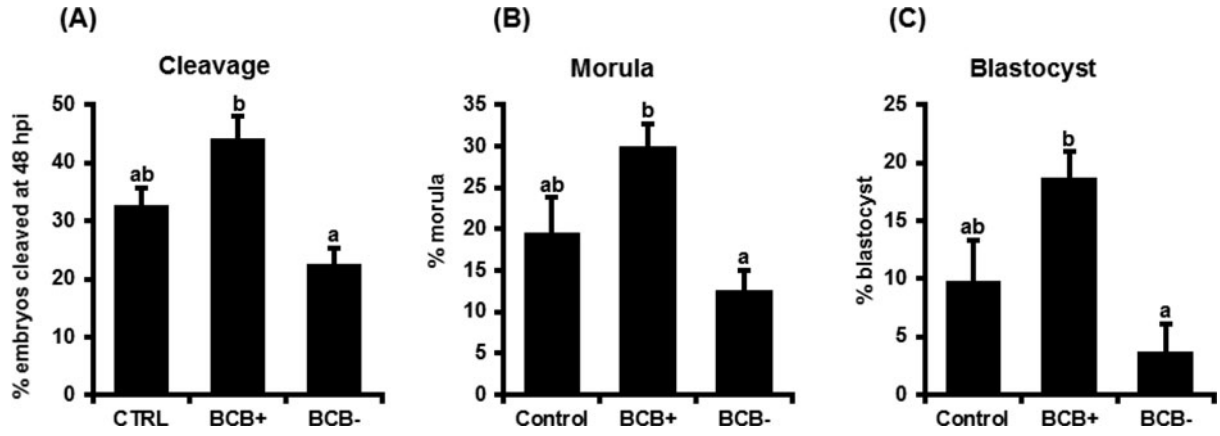

Figure 3 Effects of BCB staining on camel early embryonic development *in vitro*. Presumptive zygotes of BCB+, BCB− and control groups ($n = 4$ replicates; 20–25 zygotes) were cultured in KSOM medium until day 7. Indices of early embryonic development were recorded: (*A*) cleavage at 48 hpi; (*B*) day 5 morula; and (*C*) day 7 blastocyst. Data are shown as mean ± standard error of the mean (SEM). [a,b]Values with different superscripts across treatments indicate significant differences ($P < 0.05$).

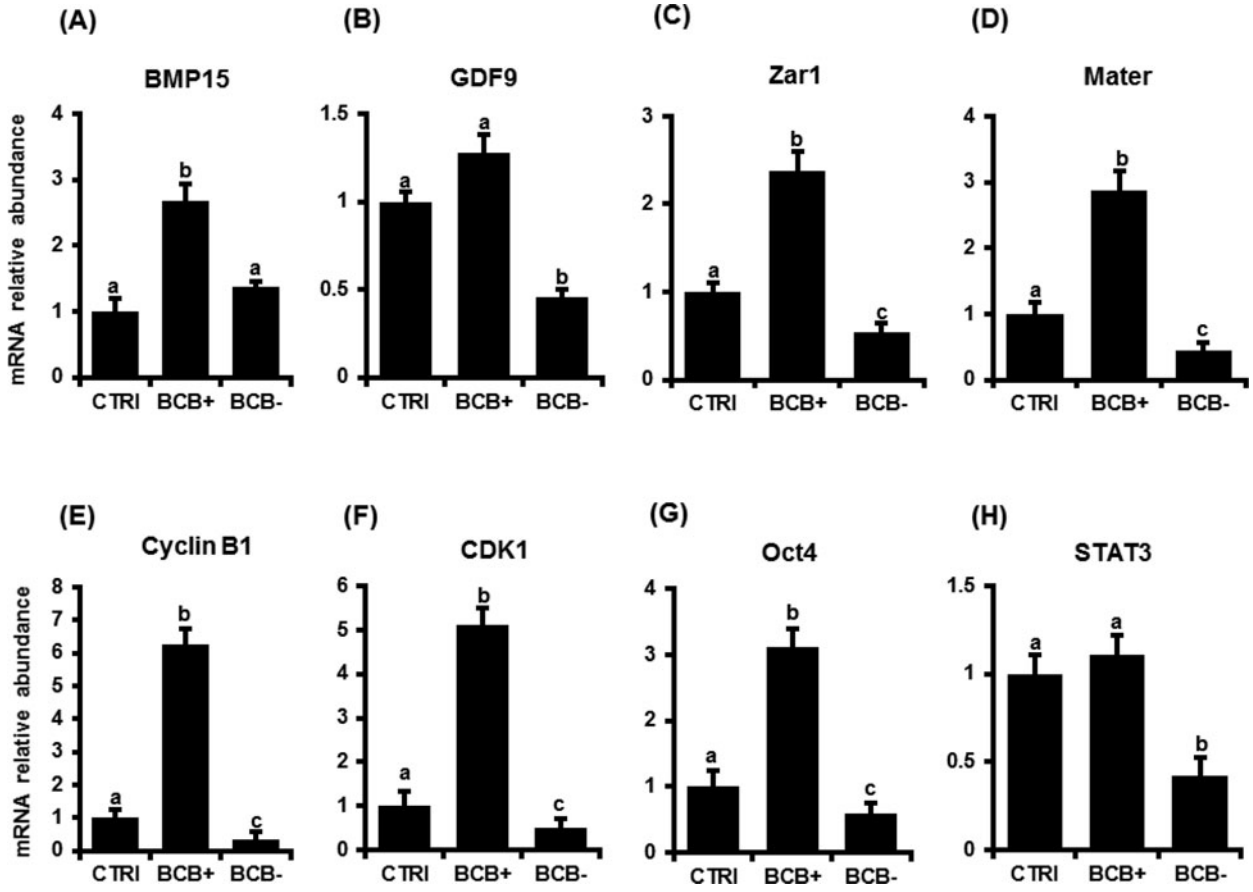

Figure 4 Expression of some candidate genes in camel oocytes screened with BCB staining. After BCB staining, GV stages oocytes ($n = 4$ replicates; 20 oocytes/group) were stripped off cumulus cells and used for RT-PCR analysis for (*A*) BMP15, (*B*) GDF9, (*C*) Zar1, (*D*) Mater, (*E*) Cyclin B1, (*F*) CDK1, (*G*) Oct4 and (*H*) STAT3. Data were normalized relative to the expression of GAPDH as endogenous control and expressed as fold change relative to control. Data are shown as mean ± standard error of the mean (SEM). [a,b,c]Values with different superscripts across treatments indicate significant differences ($P < 0.05$)

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control groups respectively (Fig. 4*E*). CDK1 was upregulated in the BCB+ group with >5-fold and 2-fold increase compared with the BCB− and control groups respectively (Fig. 4*F*). Oct4 was overexpressed in the BCB+ and control groups relative to the BCB− group, expression of Oct4 was also higher in the BCB+ group compared with controls (Fig. 4*G*). No significant difference was observed for STAT3 expression between the BCB+ and control groups, whereas both groups showed higher expression relative to the BCB− group (Fig. 4*H*). Collectively, results indicated that the BCB+ dromedary camel oocytes were enriched with several transcripts that regulate crucial functions during oocyte and follicular development.

Discussion

Although BCB staining has been widely used for screening of oocytes in several animal species, its use in camels has not been reported previously. The results of the current studies revealed that the BCB test would serve as a reliable method for selecting more competent camel oocytes for *in vitro* embryo production. BCB+ oocytes showed higher maturation, fertilization and embryonic development rates compared with BCB− and control oocytes selected solely based on morphological criteria. The transcript level of several developmentally important transcripts was higher in BCB+ GV stages oocytes.

Previous studies have demonstrated that BCB+ oocytes are more competent and yield more blastocysts compared with BCB− oocytes. It has been shown that the BCB test allowed the selection of larger oocytes with higher *in vitro* maturation and fertilization rates and an increased percentage of normally fertilized oocytes (Wang *et al*., 2012). The potential of IVF embryos derived from BCB+ oocytes to develop to morula and blastocyst stages was significantly higher compared with oocytes selected solely by morphological criteria (Rodriguez-Gonzalez *et al*., 2002; Alm *et al*., 2005; Manjunatha *et al*., 2007; Silva *et al*., 2013). In the present study, BCB+ dromedary camel oocytes showed higher maturation, fertilization rates compared with BCB− and control counterparts. Indices of early embryonic development: cleavage, development to morula and blastocyst stages, were also higher in the BCB+ group. Previous studies have demonstrated that BCB selects larger oocytes with more mitochondria and maturation-promoting factor (MPF) activity (Catalá *et al*., 2011). Conversely, less competent (BCB–) oocytes undergo fertilization failure or developmental arrest due to delayed mitochondrial DNA (mtDNA) replication that resulted from delayed onset of expression of their nuclear-encoded replication factors. The oocyte challenges to rescue this failure during the late stages of maturation. Subsequently, oocyte competence in terms of mtDNA replication and composition is not fully synchronized and oocytes fail to behave normally (May-Panloup *et al*., 2005; Spikings *et al*., 2007; Opiela *et al*., 2010; Catalá *et al*., 2013).

Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMB15) are known to play an important role during folliculogenesis and oocyte development (Chang *et al*., 2002; McNatty *et al*., 2005). Increased expression of GDF9 and BMP15 is positively correlated to oocyte and embryo developmental competence (Wu *et al*., 2007b; Li *et al*., 2014; Ashry *et al*., 2015).

Results of present studies have demonstrated the higher expression of GDF9 and BMP15 in good quality oocytes selected by BCB screening (BCB+) compared with control oocytes selected exclusively based on morphological criteria and with less competent oocytes (BCB−). ZAR1 and Mater are maternal effect transcripts that accumulate during oogenesis (Uzbekova *et al*., 2006). Previous studies have reported the higher expression of both transcripts in BCB+ ovine oocytes (Wang *et al*., 2012).

Cyclin B1 and CDK1 are implicated in the regulation of the cell cycle and resumption of meiosis in oocytes. (Castedo *et al*., 2002). Oct4 is a transcription factor known to regulate cell fate decision in early embryos (Wu & Scholer, 2014). STAT3 is also essential for early embryonic development (Teng *et al*., 2004). Previous studies (El-Sayed, 2013; El-Sayed & Ghanem, 2015) have demonstrated the higher expression of cyclin B1 and Oct4 in morphologically good quality dromedary camel oocytes.

Results of the present study demonstrated higher expression of cyclin B1, CDK1, Oct4 and STAT3 in BCB+ oocytes. Enrichment of BCB+ oocytes with the aforementioned transcripts that support several functions during oocyte maturation and/or early embryonic development explains, to some extent, the high developmental competence of BCB+ oocytes.

In conclusion, the present study provided novel information about the use of BCB staining for selection of good quality oocytes to improve the *in vitro* embryo production in the dromedary camel. Good quality camel oocytes were enriched with several transcripts that play a crucial role in folliculogenesis and oocyte development (GDF9 and BMP5), regulate the cell cycle (Cyclin B1, STAT3), are maternal effect genes required for embryo development (ZAR1 and Mater) or have transcription factor activity (OCT4).

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