Association of glucose-6-phosphate dehydrogenase activity with oocyte cytoplasmic lipid content, developmental competence, and expression of candidate genes in a sheep model

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Abstract

Purpose To evaluate associations of glucose-6-phosphate dehydrogenase (G6PDH) activity in sheep oocytes with cytoplasmic lipid content, maturational competence, developmental competence to the blastocyst stage, and gene expression of certain molecular markers.

Methods Before brilliant cresyl blue (BCB) staining test, oocytes were classified as high, middle, and low cytoplasmic lipid content (HCLC, MCLC, and LCLC) and after the test as having low or high G6PDH-activity (BCB⁺ and BCB⁻,

Capsule G6PDH-activity in sheep oocyte has been found to be related with cytoplasmic lipid content, developmental competence, and expression of quality-related genes

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respectively). After maturation in vitro, a group of oocytes were subjected to IVF followed by in vitro embryo culture and another group was used for evaluation of expression of candidate genes.

Results The cleavage and blastosyst rates were lowest ($P<0.05$) in LCLC group, intermediate ($P<0.05$) in MCLC group and highest ($P<0.05$) in HCLC group. More ($P<0.05$) oocytes in HCLC group were BCB⁺, and higher ($P<0.05$) maturation, cleavage, and blastocyst rates were seen for BCB⁺ oocytes than the BCB⁻ oocytes. Our gene expression data indicated that mRNA transcript abundance of *ITGB2*, *pZP3*, *BMP15*, and *GDF9* genes was similar between BCB oocytes groups. However, the expression of *ATP1A1* was higher ($P<0.05$) for BCB⁺ oocytes compared to BCB⁻ oocytes. In addition, *BAX* transcript abundance was similar ($P>0.05$) among BCB⁺, BCB⁻, and control groups, before and after maturation in vitro.

Conclusion Activity of G6PDH in sheep oocytes is highly associated with lipid content, and compared with the morphological parameters might be a more precise and objective predictor for subsequent developmental competence in vitro.

Keywords Lipid · G6PDH-activity · BCB · Sheep oocyte · Gene expression

Introduction

Quality of oocytes to develop into viable embryos is still an issue of major concern in assisted reproduction technologies [1–4]. In clinical practice of in vitro embryo production, morphological parameters like shape, homogeneity of cytoplasm, and compactness of cumulus cells are routinely used to identify the most competent oocytes [4]. Although such markers are

commonly used, their accuracy and precision for assessing developmental capability is highly subjective and prone to individual variation [5–7], and therefore not precisely predictive of the likelihood of subsequent blastocyst development.

Determination of glucose-6-phosphate dehydrogenase (G6PDH) activity through the brilliant cresyl blue (BCB) test [8] is one of the most under-researched methods of assessing oocyte viability and developmental competence. A function of the G6PDH enzyme is to produce ribose sugars for the synthesis of nucleic acids through the pentose shunt. As another important function, G6PDH enzyme is responsible for production of NADPH for lipid synthesis [9]. This enzyme is synthesized in oocytes during oogenesis while the follicle grows. The BCB is a dye that can be degraded by G6PDH enzyme; therefore, a large accumulation of G6PDH enzyme in growing oocyte leads to a colorless cytoplasm while cytoplasm of fully-grown oocytes remains blue due to the low level of enzyme activity [8].

Several authors [10–19], including our group [6, 20, 21], have shown a negative association between G6PDH-activity and developmental competence of oocytes. The activity of G6PDH in oocytes has been correlated with indicators of cytoplasmic maturation, such as organization of cortical granule and mitochondria [22], intracellular glutathione (GSH) level [19], and activity of the mitochondria [12, 23]. Similar to G6PDH-activity, cytoplasmic lipid content also has been shown to be highly correlated with cytoplasmic maturation, including activity of mitochondria [24, 25].

As highlighted in previous study [26], the higher lipid content of BCB⁺ bovine oocytes provides a cellular and functional basis for their greater developmental competence. If this is the case, one could speculate that the lipid content not only affects oocyte competence [25, 27, 28] but also might be an estimation of G6PDH-activity as previously shown in bovine oocytes [26]. However, to the best of our knowledge, no previous study has reported on the association between the content of cytoplasmic lipid and developmental competence of oocytes to further support early embryonic development and activity of the G6PDH enzyme.

Another important challenge in introducing a marker as a reliable indicator of oocyte quality is to understand the molecular mechanisms by which oocytes from specific categories are acquiring the higher competence to undergo preimplantation development [4]. This is probably why several authors are interested in evaluating the transcript abundance of oocytes screened for their developmental competence based on G6PDH-activity. A large number of transcripts related to developmental competence varies in abundance between oocytes of high (BCB⁺) and low (BCB[−]) G6PDH-activity [29–32]. However, the results of different studies are somewhat discordant and controversial.

With the aim of providing more empirical evidence concerning the cellular and functional basis of oocyte

competence, a prospective study was conducted using G6PDH-activity as the primary selection parameter for oocyte quality. To accomplish this we therefore, investigated the association among cytoplasmic lipid content, G6PDH-activity, and developmental competence to blastocyst stage. The enzymatic activity of G6PDH on meiotic and developmental competence was studied in detail. In addition, the expression profile of genes involved in oocyte competence (*ATP1A1*, *ITGB2*, *pZP3*, *BMP15*, and *GDF9*) and apoptosis (*BAX*) in oocytes with different G6PDH-activity was studied.

Materials and methods

Chemicals and supplies

All products were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA) unless stated otherwise.

Oocyte collection

Ovaries were collected from ewes at a local slaughterhouse and transported to the laboratory within 2–3 h in saline solution at a temperature of approximately 35–37 °C. Afterwards, cumulus-oocyte complexes (COCs) were isolated from follicles using the slicing method. Only oocytes completely surrounded by compact and thick cumulus were randomly selected and used for the experiments [33].

Oocyte cytoplasmic lipid content and Oil Red O (ORO) staining

Immature COCs were visualized under a stereomicroscope for evaluation of their cytoplasmic lipid content. The COCs were divided into three groups based on cytoplasm morphology, 1) high cytoplasmic lipid content (HCLC), 2) middle cytoplasmic lipid content (MCLC), and 3) low cytoplasmic lipid content (LCLC), as shown in Fig. 1a to c, respectively.

Lipid accumulation score was evaluated in oocytes using Oil Red O (ORO) staining technique. For this purpose, before IVM, COCs from each group of HCLC, MCLC, and LCLC were completely denuded by gently pipetting, and fixed in 4 % paraformaldehyde. Fixed oocytes were stained for 3 min with 0.5 % ORO dissolved in 60 % isopropanol, mounted on slide glasses, covered with a coverslip, and imaged under a bright-field microscope as shown in Fig. 1d to f.

BCB staining

The COCs were exposed to 26 mM of BCB dye diluted in modified phosphate buffer saline (mPBS) for 90 min at 38.5 °C in humidified air atmosphere [6, 21, 34, 35].

Fig. 1 Classification of COCs based on cytoplasmic lipid content under a stereomicroscope. Typical images of oocytes with (a) high cytoplasmic lipid content (HCLC), (b) middle cytoplasmic lipid content (MCLC), and (c) low cytoplasmic lipid content (LCLC). Lipid accumulation score of

oocytes evaluated following ORO staining under a stereomicroscope. The figure shows typical image of each group of cumulus-free immature oocytes after ORO staining: (d) HCLC, (e) MCLC, and (f) LCLC. (Scale bar=50 μ m)

Following BCB staining, COCs were washed 3 times in mPBS. After washing, COCs were observed under stereomicroscope and classified into two groups based on their cytoplasm coloration: COCs with blue color in cytoplasm considered as BCB^+ group and COCs with colorless cytoplasm detected as BCB^- group (Fig. 2). A group of COCs was randomly assigned as a control group and was transferred to maturation medium without being exposed to the BCB dye.

In vitro maturation

COCs were washed three times in maturation medium consisting of bicarbonate-buffered TCM-199 with 2 mM L-glutamine supplemented with 10 % fetal bovine serum, 5.5 mg mL⁻¹ sodium pyruvate, 25 μ g mL⁻¹ gentamycin sulphate, 5.0 μ g mL⁻¹ LH, 0.5 μ g mL⁻¹ FSH, and 1 μ g mL⁻¹ estradiol. In vitro maturation (IVM) was performed by culturing approximately 10 COCs for 24 h in 50 μ l maturation medium droplet under mineral oil at 39 °C, 5 % CO₂ in a humidified atmosphere.

Nuclear chromatin evaluation

After IVM, COCs were freed from surrounding cumulus cells, stained with 2.5 mg mL⁻¹ Hoechst 33258 in 3:1 (v/v) glycerol/PBS, and kept until observation. Oocytes were then evaluated in relation to their meiotic stage under

epifluorescence microscope (Nikon, Tokyo, Japan) and classified as being in germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase-I (MI), or metaphase-II (MII) stages, as previously described [6, 21].

In vitro fertilization

After IVM, groups of 10 COCs were washed two times in HEPES-buffered synthetic oviductal fluid (HSOF) and placed in 44 μ l drops overlaid with equilibrated mineral oil. Fertilization medium was SOF supplemented with 4 IU mL⁻¹ heparin, PHE (20 μ M penicillamine, 10 μ M hypotaurine, 1 μ M

Fig. 2 Oocytes with different G6PDH-activities; BCB^+ oocytes (blue cytoplasm due to low G6PDH-activity) indicated by blue arrows and BCB^- oocytes (colorless cytoplasm due to high G6PDH-activity) illustrated by grey arrows [6]

epinephrine), and 2 % (v/v) sheep serum [36]. A frozen sperm was thawed, washed, and a percoll gradient used to separate a high quality viable sperm sample that was added to oocytes at a concentration a 2×10^6 spermatozoa mL^{-1} in fertilization drops. The COCs and sperm were incubated together for approximately 18 h under the same conditions as described for maturation above.

In vitro culture

Following fertilization in vitro, denuded presumptive zygotes were washed three times in HEPES-buffered synthetic oviductal fluid (HSOF) medium. Groups of 15 to 20 zygotes were cultured in 30 μL culture drops of SOFaa (2 % BME-essential amino acids, 1 % MEM-nonessential amino acids) supplemented with 8 mg mL^{-1} bovine serum albumin, 1 mM glutamine, 0.34 mM tri-sodium citrate, and 2.77 mM myo-inositol under equilibrated mineral oil until day 9 post-fertilization [36].

RNA extraction, cDNA synthesis and real time PCR

Total cellular RNA was extracted using TRI-reagent. Synthesis of cDNA was carried out with M-MuLV reverse transcriptase (RT) and random hexamer as primer, according to the manufacturer's instructions (Fermentas; St. Leon-Rot, Germany). Real-Time quantitative reverse transcription PCR (RT-PCR) reactions of 25 μL were conducted in Rotor Gene 6000 (Corbett Life Science, Sydney, Australia), by adding 12.5 μL 2X SYBR Premix Ex Taq, 0.4 μL of final concentration for each primer, 2 μL template, and distilled water to reach the volume of 25 μL . Real-time PCR was performed in two steps with the following thermal setting: 3 min at 95 °C for initial enzyme activation followed by 40 amplification cycles (each 5 s at 95 °C, and 20 s at 60 °C with fluorescence detection), and a final step of melting curve analysis. The information for primers used for real-time PCR is listed in Table 1. All the samples were analyzed in duplicate, and the average value of the duplicate was used for quantification. The data were normalized to *GAPDH* and $2^{-\Delta\Delta\text{Ct}}$ methodology was used for relative quantification [37].

Experimental design

Experiment 1: Cytoplasmic lipid content in relation to lipid accumulation score and subsequent embryo development potential

Based on their cytoplasmic lipid content, COCs were categorized into three groups of HCLC, MCLC, and LCLC. To ensure the classification accuracy, lipid accumulation score was evaluated in each group of HCLC, MCLC, and LCLC oocytes using ORO staining technique. For this, before IVM,

25 to 30 COCs from each group were stained with ORO and were imaged individually. The images were then analyzed with ImageJ software (<http://rsb.info.nih.gov/ij>) to quantify objectively the amount of lipid accumulation and to validate the previous subjective visual score evaluation given to the COCs.

Moreover, to determine whether cytoplasmic lipid content of the COCs has a positive predictive value for developmental competence, COCs obtained from groups of HCLC ($n=91$), MCLC ($n=87$), and LCLC ($n=96$) were matured and fertilized in vitro, and the subsequent developmental capacity, i.e. cleavage and blastocyst rates for each group was recorded at day 3 and day 9 post-insemination, respectively.

Experiment 2: Cytoplasmic lipid content in relation to G6PDH-activity

To study the relationship between cytoplasmic lipid content and G6PDH-activity in COCs, proportion of BCB^+ and BCB^- oocytes was recorded for each group of HCLC, MCLC, and LCLC after the BCB test. COCs of HCLC ($n=242$), MCLC ($n=225$), and LCLC ($n=222$) groups were stained with BCB dye. After staining, COCs from each group of HCLC, MCLC, and LCLC were examined on a stereomicroscope. Based on the G6PDH-activity, COCs of each group were further classified as having low (BCB^+) or high (BCB^-) G6PDH-activity.

Experiment 3: G6PDH-activity in relation to oocyte meiotic competence and embryo developmental potential

In order to evaluate the effect of G6PDH-activity on meiotic competence, after collection, the COCs were categorized into BCB^+ ($n=203$) and BCB^- ($n=188$) groups and then matured in vitro. Nuclear status was determined after maturation culture for each group after fixing and staining with Hoechst.

Moreover, to determine the predictive value of G6PDH-activity for developmental competence, matured COCs obtained from BCB^+ ($n=199$) and BCB^- ($n=187$) groups were fertilized in vitro, and rates of cleavage and development to the blastocyst stage were recorded at days 3 and 9 post-insemination, respectively.

Experiment 4: G6PDH-activity in oocytes in relation to the expression of candidate genes

Interactions between G6PDH-activity and expression of developmentally important genes (Table 1) were examined in immature oocytes using RT-PCR assays.

For both BCB^+ and BCB^- groups, three biological replicates, each containing ten immature COCs, were analyzed after being freed from surrounding cumulus cells. Oocytes were then subjected to RNA isolation and relative quantification of *ATP1A1*, *ITGB2*, *pZP3*, *BMP15* and *GDF9* transcripts.

Table 1 Details of primers used for real time PCR quantitative analysis

Gene name	Primer sequences	Accession number	Annealing temperature (°C)	Product size (bp)
<i>ATP1A1</i>	F: 5'-CTGCCGTCGTCATCATAAC-3' R: 5'-TCACTTCCACCAGGTCTCC-3'	NM_001009360	61	171
<i>ITGB2</i>	F: 5'-GAGCAGTCCTTACCATCC-3' R: 5'-GGCACTCGCAGTTCTTCC-3'	NM_001009485	62	184
<i>pZP3</i>	F: 5'-CTGATGGAGGAGAACTGGAG-3' R: 5'-GACTTGAGCCTGGAGGTG-3'	XM_004020981	60	81
<i>BMP15</i>	F: 5'-AAGGCCTCTCAGAGGCTC-3' R: 5'-GAGAGGTGGGAATGAGTTAGG-3'	NM_001114767	61	132
<i>GDF9</i>	F: 5'-GCAGCCAGATGACAGAGC-3' R: 5'-CTGATGGAAAGGTTCTTGC-3'	NM_001142888	61	176
<i>BAX</i>	F: 5'-TGGAGATGAATTGGACAGTAAC-3' R: 5'-GCCTTGAGCACCAGTTTG-3'	XM_004015363	61	174
<i>GAPDH</i>	F: 5'-GGAGGGACTTATGACCACTG-3' R: 5'-CTGCTTACCACCTTCTTG-3'	NM_001190390	60	135

PCR, polymerase chain reaction; F, forward; R, reverse

The expression in BCB⁺ oocytes was compared with the expression in BCB⁻ oocytes.

Moreover, the expression of apoptosis-related *BAX* gene in both immature and mature oocytes was compared between BCB⁺ and BCB⁻ groups. For this also three pools of biological replicates each containing ($n=10$) immature/mature oocytes were used for total RNA isolation. To evaluate effect of BCB staining on induction of apoptosis, expression of *BAX* gene in BCB treated oocytes was compared with COCs that were not exposed to the BCB dye (and were assigned to control group).

Statistical analyses

The score of images for HCLC, MCLC, and LCLC oocytes were analyzed by one-way ANOVA followed by Tukey's pairwise comparison. The proportion of COCs with high, middle, and low cytoplasmic contents, the oocyte nucleus status after IVM, and the cleavage and blastocyst rates after fertilization were compared by chi-square analysis. Relative

gene expression levels of different genes were analyzed by ANOVA. A probability of $P<0.05$ was considered to be significant.

Results

Experiment 1: Cytoplasmic lipid content in relation to lipid accumulation score and subsequent embryo development potential

As expected the lipid accumulation score of oocytes was different ($P<0.05$) among HCLC, MCLC, and LCLC groups (Fig. 3). The lipid accumulation score of oocytes with HCLC (26.2 ± 2.5 ; $n=27$) was greater ($P<0.05$) than those of MCLC (20.5 ± 2.9 ; $n=30$), and LCLC (13.5 ± 3.9 ; $n=25$) oocytes. Moreover, the lipid accumulation score was different ($P<0.05$) between MCLC and LCLC oocytes (Fig. 3).

A higher ($P<0.05$) cleavage rate was observed for HCLC (72.5 %) versus LCLC (44.8 %) oocytes (Table 2). However, the cleavage rate of MCLC oocytes was similar ($P>0.05$) to both HCLC and LCLC oocytes. A higher ($P<0.05$) blastocyst rate was obtained for HCLC oocytes than for both MCLC, and

Fig. 3 Lipid accumulation score of HCLC, MCLC, and LCLC oocytes analyzed with ImageJ Software. ^{a,b,c} Bars with different letters represent groups that were different ($P<0.05$)

Table 2 Relationship between cytoplasmic lipid content and embryo development in sheep oocytes matured in vitro and subjected to in vitro fertilization (cumulative results of three replicates)

Group	No. COCs	Embryo development	
		Cleaved % (n)	Blastocyst % (n)
HCLC	91	72.5 (66) ^a	36.3 (33) ^a
MCLC	87	60.9 (53) ^{ab}	17.2 (15) ^b
LCLC	96	44.8 (43) ^b	6.3 (6) ^c

^{a,b,c} Within columns different superscripts were statistically different ($P<0.05$)

Table 3 Interaction between cytoplasmic lipid content and G6PDH-activity in sheep oocytes before in vitro maturation (cumulative results of three replicates)

Group	No. COCs	BCB coloration	
		BCB ⁺ % (n)	BCB ⁻ % (n)
HCLC	242	82.4 (197) ^a	18.8 (45) ^a
MCLC	225	56.4 (127) ^b	43.6 (98) ^b
LCLC	222	12.2 (27) ^c	87.8 (195) ^c

^{a,b,c} Within columns different superscripts were statistically different ($P<0.05$)

LCLD oocytes. Oocytes in the MCLC group also produced more ($P<0.05$) blastocysts than the LCLC oocytes.

Experiment 2: Cytoplasmic lipid content in relation to G6PDH-activity

The proportion of BCB⁺ oocytes within the HCLC group (82.4 %) was higher ($P<0.05$) than within both the MCLC, and LCLD groups (56.4 % and 12.2 %, respectively; Table 3). At the same time, the HCLC group (18.8 %) had lower ($P<0.05$) proportion of BCB⁻ oocytes compared with the MCLC, and LCLC groups (43.6 % and 87.8 %, respectively). Moreover, more ($P<0.05$) BCB⁺ oocytes and fewer ($P<0.05$) BCB⁻ oocytes were observed in MCLC group than in LCLC group.

Experiment 3: G6PDH-activity in relation to oocyte meiotic competence and embryo developmental potential

More ($P<0.05$) BCB⁺ oocytes (77.8 %) reached MII stage compared to BCB⁻ group (52.1 %; Table 4). The rate of oocytes with MI nuclear status observed after IVM was lower ($P<0.05$) in BCB⁺ group (20.2 %) than BCB⁻ group (40.4 %). The rates of oocytes with GV and GVBD nuclear status did not differ ($P>0.05$) between the groups. After IVM, oocytes from BCB⁺ and BCB⁻ groups were fertilized in vitro. Proportional data for cleavage and development to blastocysts are summarized in Table 5. Cleavage rate (73.9 % vs. 49.2 %) and

Table 4 G6PDH-activity in sheep oocytes in relation to nuclear status after in vitro maturation (cumulative results of three replicates)

Group	No. oocytes	Nuclear status			
		GV % (n)	GVBD % (n)	MI % (n)	II % (n)
BCB ⁺	203	0 (0)	2.0 (4)	20.2 (41) ^a	77.8 (158) ^a
BCB ⁻	188	2.1 (4)	5.3 (10)	40.4 (76) ^b	52.1 (98) ^b

^{a,b} Within columns different superscripts were statistically different ($P<0.05$)

Table 5 G6PDH-activity in sheep oocytes in relation to embryo development after in vitro fertilization (cumulative results of three replicates)

Group	No. COCs	Embryo development	
		Cleaved % (n)	Blastocyst % (n)
BCB ⁺	199	73.9 (147) ^a	35.2 (70) ^a
BCB ⁻	187	49.2 (92) ^b	4.8 (9) ^b

^{a,b} Within columns different superscripts were statistically different ($P<0.05$)

blastocyst rate (35.2 % vs. 4.8 %) were higher ($P<0.05$) for BCB⁺ oocytes than BCB⁻ oocytes.

Experiment 4: G6PDH-activity in oocytes in relation to the expression of candidate genes

The expression of *ATP1A1* was higher ($P<0.05$) for BCB⁺ immature oocytes when compared with BCB⁻ immature oocytes. However, the relative transcript abundance of *ITGB2*, *pZP3*, *BMP15*, and *GDF9* genes was not different ($P>0.05$) between BCB⁺ and BCB⁻ immature oocytes (Fig. 4).

Moreover, no differences ($P>0.05$) were detected for *BAX* transcripts among BCB⁺, BCB⁻, and control groups (Fig. 5), either in immature or mature oocytes.

Discussion

Cytoplasmic lipid content has been described recently as a determinant parameter for oocyte development competence and early embryo development in cattle and horses [25, 27]. However, to date, this relationship has not been reported yet for sheep oocytes. Therefore, initially, we investigated herein the associations between cytoplasmic lipid content, lipid accumulation score, and capacity of sheep oocytes to develop to

Fig. 4 Relative mRNA expression of *ATP1A1*, *ITGB2*, *pZP3*, *BMP15* and *GDF9* transcripts of immature BCB⁺ oocytes compared with BCB⁻ oocytes. ^{a,b} Bars with different letters represent groups that were different ($P<0.05$)

Fig. 5 Relative mRNA expression of *BAX* transcripts before (hour 0) and after IVM (hour 24) of BCB⁺ and BCB⁻ oocytes in comparison with the control group. No differences ($P > 0.05$) were detected among groups

blastocyst stage, highlighting the importance of lipid deposition in oocytes. The ORO staining is a semi-quantitative method to evaluate the lipid content, which by definition generates results with arbitrary precision. Using ORO we studied the association between cytoplasmic lipid content and lipid accumulation score. As predicted, there were significant differences among the HCLC, MCLC, and LCLC groups in overall scale scores, with those of the HCLC group being highest and those of the LCLC group being the lowest. Next, we investigated the association between oocyte cytoplasmic lipid content and subsequent embryo development in vitro. Our data demonstrated that high lipid content in sheep oocytes is positively associated with elevated cleavage rate after fertilization and blastocyst formation. Our data supported the hypothesis that HCLC oocytes have higher developmental competence than the LCLC oocytes. In a previous study by Jeong et al. (2009), bovine oocytes were classified in three color groups (e.g., pale color, brown color, and dark color), depending on the color of cytoplasm and lipid content [25]. These authors found an association between cytoplasmic color and developmental efficiency of bovine in vitro-produced embryos, with the dark color group producing the most competent oocytes and the pale group the least. The bovine findings are in agreement with our results using sheep oocytes.

The higher developmental competence observed in HCLC oocytes compared to MCLC and LCLC oocytes raised the question whether differences in cytoplasmic lipid content might have been reflected in G6PDH-activity as well. Our data demonstrated that high G6PDH-activity in sheep BCB⁻ oocytes was strongly associated with decreased cytoplasmic lipid content. The high proportion of BCB⁺ oocytes in the HCLC group may be an explanation for critical role of cytoplasmic lipid in acquisition of developmental competence. This is in accordance with recent findings [26] showing that oocytes with a relatively low capacity to metabolize BCB (BCB⁺ oocytes) contained more cytoplasmic lipid than those that still had G6PDH-activity sufficient to reduce BCB (BCB⁻ oocytes).

As oocytes grow, excess lipid is stored within the cytoplasm [38]. This lipid accumulation is used by mitochondria for increased production of ATP required for oocyte maturation and subsequent development [39]. Our previous studies have shown that BCB⁺ oocytes had a greater diameter [6] and volume [21] than the BCB⁻ oocytes. The greater diameter of BCB⁺ oocytes and presumably the larger sizes of their follicles of origin show that this subgroup of oocytes originated from more advanced follicular stages than the BCB⁻ oocytes [6, 11, 21, 40]. Therefore, the larger diameters of BCB⁺ oocytes may indeed have been the most plausible explanation for their greater lipid storage capacity, as seen in this study.

The value of the use of G6PDH-activity in selection of the most competent oocytes has been evaluated herein. COCs were classified based on their G6PDH-activity, followed by maturation, fertilization, and subsequent culture of the resultant embryos up to the blastocyst stage. The rate of MII oocytes 24 h after IVM was higher in the BCB⁺ group than in the BCB⁻ group. Furthermore, the blastocyst rate of BCB⁺ oocytes was higher compared with the BCB⁻ oocytes 9 days post-fertilization. The better performance of BCB⁺ oocytes compared with BCB⁻ oocytes might have been related to the optimal follicular development during the final phases of folliculogenesis [6, 11]. Hence, higher cytoplasmic lipid content and lower G6PDH-activity prior to IVM can be proposed as an ideal marker for induction of cytoplasmic maturation and to obtain the best maturation rates. Therefore, this study demonstrates that BCB⁺ oocytes are intrinsically more competent to complete normal developmental processes successfully.

Incompetency of BCB⁻ versus BCB⁺ oocytes has been related to some intrinsic parameters such as mitochondrial activity [12, 23, 32], mitochondrial distribution [19, 22], mitochondrial DNA copy number [41], GSH content [19], and as well as relative abundance of gene transcripts [29, 32]. The difference in abundance of transcripts related to maturation and embryo development between BCB⁺ and BCB⁻ groups has been a topic of several reports on the literature [12, 21, 29, 32, 42–46]. Nevertheless, in spite of broad information of molecular markers on oocyte developmental competence in various animal models, limited data is available for sheep [12, 47]. The present study investigated the expression of candidate genes involved in developmental competence of oocytes, which have been screened based on both G6PDH-activity and cytoplasmic lipid content. Transcript abundance was evaluated between the two oocyte groups which represented high (BCB⁺) and low (BCB⁻) developmental competence. The genes investigated in this work were related to metabolism (*ATPIA1*), sperm receptor (*pZP3*), cell surface adhesion (*ITGB2*), and growth factors (*BMP15* and *GDF9*). In spite of the higher embryo development observed in BCB⁺ oocytes compared to BCB⁻ oocytes, the two oocyte groups were not different in relative expression in any of the genes studied, except for *ATPIA1* gene. *ATPIA1* expression was reduced in the BCB⁻ oocytes compared with

BCB⁺ oocytes. The crucial role of *ATP1A1* gene for developmental competence [47] and its importance during blastulation has been previously reported [48]. In contrast to our study, Catala et al. (2011) observed no difference in expression of *ATP1A1* gene in relation to BCB classification using cattle oocytes [12]. However, in agreement with our results, Somfai et al. (2011) reported low expression of *ATP1A1* associated with low blastocyst formation rate. *ATP1A1*, the alpha subunit of the plasma membrane NA⁺/K⁺ATPase has an important function in oocyte energy metabolism [48]. Furthermore, *ATP1A1* is reported to play roles in blastocyst formation in mammals [48]. Reduced level of maternal *ATP1A1* mRNA in BCB⁻ oocytes may either directly limit blastocyst formation or at least reflect developmental competence of embryos to the blastocyst stage. Therefore, we consider this gene to be a possible marker for quality and developmental competence in sheep oocytes.

It is known that oocyte apoptosis is likely to be influenced by multiple extrinsic and intrinsic inputs [49]. Because the COCs are exposed to a dye in the BCB test, induction of apoptosis in oocytes could be a matter of considerable concern. In this study we evaluated the relative abundance of *BAX* gene, which is a member of the *Bcl-2* gene family and considered to be an apoptotic gene. No significant differences in *BAX* transcript levels were detected between BCB⁺, BCB⁻, and control group (no dye exposure), either before maturation or 24 h after the onset of maturation in vitro. In contrast to our results, Opiela et al. (2008) reported that bovine oocytes subjected to the BCB test tended to be more apoptotic; however the results at the protein level did not confirm that conclusion [44]. In another study by Su et al. (2012), bovine BCB⁺ embryos had fewer apoptotic cells than BCB⁻ embryos and the expression of the pro-apoptotic gene *BAX* was down-regulated in BCB⁺ blastocysts [50]. In light of these contradictory results, additional studies should be performed to confirm the non-invasive nature of the BCB test regarding cell apoptosis in oocytes and embryos of ewes.

In summary, oocytes with high cytoplasmic lipid content had less G6PDH-activity and higher developmental potential in ewes. Our data not only demonstrate the interaction of cytoplasmic lipid content and G6PDH-activity, but also highlight a more accurate application of the BCB staining test for screening the most competent oocytes.

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