Brilliant Cresyl Blue stain selects largest oocytes with highest mitochondrial activity, maturation-promoting factor activity and embryo developmental competence in prepubertal sheep

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Abstract

The aim of this study was to test the Brilliant Cresyl Blue (BCB) stain to select prepubertal sheep oocytes for *in vitro* blastocyst production. Oocyte diameter, mitochondrial activity, maturation-promoting factor (MPF) activity and mRNA relative expression (RE) of genes related to metabolism (ATPase Na⁺/K⁺ transporting α 1 (*ATP1A1*) and cytochrome c oxidase subunit 1 (*COX1*)) and constitutive function of the cell (cytoplasmic polyadenylation-element-binding protein (*CPEB*) and *S100A10*) were assessed. Immature oocytes were exposed to different BCB concentrations (13, 26, 39 and 52 μ M) and classified according to their cytoplasm colouration as grown BCB+ (blue cytoplasm) and growing BCB- (colourless cytoplasm). Staining oocytes with 13 μ M BCB during 60 min allows selection of (BCB+) the largest (123.66 μ m) and most competent oocytes to develop to the blastocyst stage (21%) with a higher number of cells (69.71 ± 6.19 S.E.M.) compared with non-stained BCB- oocytes (106.82 μ m, 9% and 45.91 ± 3.35 S.E.M. respectively). Mitochondrial activity, assessed by MitoTracker Orange CMTMRos probe, was significantly higher in BCB+ than in BCB- oocytes after *in vitro* maturation (3369 and 1565 AU respectively). MPF activity was assessed by CDC2 kinase activity assay showing significantly higher activity at metaphase II stage in BCB+ than in BCB- oocytes (1.479 ± 0.09 and 1.184 ± 0.05 optical density respectively). The genes analysed in this work, *ATP1A1*, *COX1*, *CPEB* and *S100A10*, did not show significant effect in mRNA RE between BCB selected oocytes. In conclusion, BCB stains larger and more competent oocytes to develop to the blastocyst stage with more active mitochondria and MPF activity and higher blastocyst cell number.

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Introduction

In vitro embryo production is closely related to oocyte source and quality (Rizos *et al.* 2002, Cognie *et al.* 2003). Thus, the efficiency of *in vitro* techniques is low when using prepubertal animals as oocyte donors. Prepubertal oocytes are characterised as having abnormal cytoplasmic maturation and lower ability to achieve the blastocyst stage than those coming from adult donors (Armstrong 2001). This has been shown in cattle (Revel *et al.* 1995), sheep (O'Brien *et al.* 1996) and pigs (Peters *et al.* 2001). Ovaries from prepubertal animals have a high percentage of antral follicles with a diameter smaller than 3 mm (Martino *et al.* 1994), making it difficult to release the cumulus–oocyte complexes (COCs) by traditional aspiration. For this reason, oocytes are routinely obtained by slicing the ovary surface, resulting in oocytes with heterogeneous diameter,

different COC morphology and at varying stages of atresia. It is known that there is a direct and positive relationship among follicle size, oocyte diameter and embryo development (Gilchrist *et al.* 1995, Barnes & Sirard 2000). In prepubertal goats, we have previously shown that oocytes with a diameter larger than 125 μ m produced higher percentages of blastocyst after IVF (Anguita *et al.* 2007) and ICSI (Jimenez-Macedo *et al.* 2007) and oocytes coming from follicles larger than 3 mm develop to the blastocyst stage in a significantly higher percentage than oocytes from follicles smaller than 3 mm (Romaguera *et al.* 2010). Brilliant Cresyl Blue (BCB) stain is known to be a non-invasive methodology that allows the selection of oocytes with larger diameters among a heterogeneous pool. The BCB test determines the intracellular activity of glucose-6-phosphate dehydrogenase (G6PDH), a pentose phosphate pathway enzyme that gradually decreases its activity as oocytes

reach their growth phase. BCB dye can be reduced by G6PDH activity, therefore oocytes that have reached their growth phase cannot reduce BCB to a colourless compound and exhibit a blue coloured cytoplasm (BCB+). However, growing oocytes are expected to have a high level of G6PDH activity and be able to reduce the blue compound, resulting in a colourless oocyte cytoplasm (BCB-). In our previous studies in prepubertal goats (Rodriguez-Gonzalez *et al.* 2002) and cows (Pujol *et al.* 2004), we have shown the usefulness of the BCB stain to select the larger and more competent oocytes for *in vitro* blastocyst production.

Blastocyst viability is related to the timing of blastocyst formation (Majerus *et al.* 2000), embryo cryotolerance assessed by blastocyst re-expansion rates post-warming (Leoni *et al.* 2009) and the number of blastomeres at a given age and their allocation to the inner cell mass (ICM) and the trophectoderm (TE; Papaioannou & Ebert 1988). The blastocyst is composed of two different cell lineages: TE and the ICM. The inside cells develop into the ICM of the blastocyst and the outside cells progressively lose their pluripotency, differentiating into an extraembryonic tissue, the TE.

Mitochondria are maternally inherited organelles that use oxidative phosphorylation to supply energy (ATP) to the cell (Stojkovic *et al.* 2001). The distribution of mitochondria changes during oocyte maturation and fertilisation with the aim of bringing mitochondria to the region of the cell where a higher level of ATP (Van Blerkom & Runner 1984) or calcium (Sousa *et al.* 1997) is required. It has been demonstrated that mitochondrial function and the cytoplasmic ATP level can affect fertilisation, resulting in a significant increase in blastocyst rates or their total failure after IVF (Van Blerkom *et al.* 1995, Liu *et al.* 2000). Mitochondrial distribution and activity are modified during oocyte *in vitro* maturation (IVM) and this differs among species such as cattle (Stojkovic *et al.* 2001, Tarazona *et al.* 2006), dogs (Valentini *et al.* 2010), goats (Velilla *et al.* 2006), horses (Torner *et al.* 2007), humans (Van Blerkom *et al.* 1995, 2008, Dell'Aquila *et al.* 2009), mice (Calarco 1995) and pigs (Torner *et al.* 2004, Brevini *et al.* 2005). Using the fluorescence probe MitoTracker Green, Sun *et al.* (2001) concluded that *in vitro* matured pig oocytes present changes in the distribution of mitochondria causing the incomplete movement of mitochondria into the inner cytoplasm affecting the cytoplasmic maturation. In our laboratory, we found differences in the distribution pattern of mitochondria between adult and prepubertal goat oocytes (Velilla *et al.* 2006).

Meiosis and mitosis are regulated by the activity of the maturation-promoting factor (MPF). This universal cell cycle regulator is a heterodimer protein composed of two subunits, the catalytic subunit p34^{cdc2} (serine–threonine kinase activity) and the regulatory subunit cyclin B1. The association of these two subunits is a requirement for the activation of the protein kinase

activity; also the phosphorylation of p34^{cdc2} on threonine 161 by the protein kinase CDC2-activation kinase (CAK) and dephosphorylation on threonine 14 and tyrosine 15 by CDC25 phosphatase is necessary. MPF activity appears just before germinal vesicle breakdown (GVBD) increasing until metaphase I; its activity is decreased in anaphase–telophase while its maximum level is reached at metaphase II (MII). It has been shown that incompetent goat oocytes have a limited amount of cyclin B1 (Hue *et al.* 1997) and p34^{cdc2} (Anguita *et al.* 2007). MPF activity in calf and lamb oocytes were significantly lower than in cow and ewe oocytes (Ledda *et al.* 2001, Salamone *et al.* 2001), whereas (Han *et al.* 2010) showed in mice that the MPF activity of prepubertal oocytes was significantly higher than that of adult oocytes. In prepubertal goats, Anguita *et al.* (2007) showed higher MPF activity and oocyte competence to develop up to the blastocyst stage in oocytes with a diameter larger than 135 µm. In conclusion, MPF activity could be a useful tool in analysing differences in oocyte quality.

Competence is acquired during oocyte growth, when the synthesis and storage of proteins and RNA take place (Crozet *et al.* 1981, Brevini-Gandolfi & Gandolfi 2001). The mRNA content in oocytes is affected by animal nutrition (Pisani *et al.* 2008), follicle diameter (Caixeta *et al.* 2009), IVM culture media (Salhab *et al.* 2011), *in vivo* and *in vitro* conditions (Wells & Patrizio 2008) and apoptosis (Li *et al.* 2009). Thus, mRNA stored in oocytes could represent a valuable tool as a molecular marker for oocyte quality. In this study, we decided to analyse the expression of two genes involved in metabolism (ATPase Na⁺/K⁺ transporting alpha 1 (ATP1A1) and cytochrome c oxidase subunit 1 (COX1)) and two genes involved in the constitutive function of the cell (cytoplasmic polyadenylation-element-binding protein (CPEB) and calcium-binding protein (S100A10)).

To our knowledge, there are no reports regarding *in vitro* developmental competence of prepubertal sheep oocytes selected by the BCB test. The aim of this study was to evaluate the BCB test as an indirect measure of oocyte growth to select more competent lamb oocytes for IVM, IVF and embryo culture. Also, we aimed to assess oocyte diameter, mitochondrial activity and distribution assessed by MitoTracker Orange CMTMRos probe, the MPF activity and the relative mRNA expression of four maturation gene candidates by real-time PCR in BCB selected oocytes.

Results

Embryo development of prepubertal sheep oocytes selected with different BCB concentrations

The percentage of BCB+ obtained after staining with different concentrations of BCB was 19, 28, 36 and 47% for 13, 26, 39 and 52 µM BCB respectively (Table 1).

Table 1 Immature prepubertal sheep oocytes exposed at different concentrations of Brilliant Cresyl Blue (BCB).

BCB concentration (μM)	Total COC	Oocyte classification	
		BCB+, n (%)	BCB-, n (%)
13	226	44 (19) ^{a,A}	182 (81) ^{a,B}
26	225	64 (28) ^{b,A}	161 (72) ^{b,B}
39	234	85 (36) ^{b,A}	149 (64) ^{b,B}
52	283	132 (47) ^c	151 (53) ^c

Values in the same column (^{a,b,c}) or row (A, B) with different letters differ significantly (Fisher test; $P < 0.05$).

Although staining with 13 μM BCB showed a low percentage of stained oocytes (BCB+), the number of blastocysts obtained in this group (21%) was significant higher ($P < 0.05$) than with 39 μM (10%) and 52 μM BCB (8%; Table 2). Of 174 inseminated oocytes from the control group (not exposed to BCB), 116 (67%) were cleavage oocytes and 14 (8%) reached the blastocyst stage. This percentage of blastocysts was significantly different from BCB+ but not from BCB- oocytes. After 24 h of IVM there were no significant differences in the percentage of oocytes (stained with 13 μM BCB) reaching the MII stage in BCB+, BCB- and the control group (86, 72.5 and 80% respectively). After 17 h of IVF, the percentage of normal fertilisation (2PN) was significantly different ($P < 0.05$) between the BCB+ (40%) and BCB- groups (22%), and between BCB+ and controls (23%) selected with 13 μM BCB (Table 3).

The analysis of the cell number counting at day 8 post-insemination of all blastocysts produced *in vitro* from prepubertal sheep oocytes selected with 13 μM BCB is summarised in Table 4. BCB+ oocytes produced blastocysts with a significantly ($P < 0.001$) higher number of cells than BCB- oocytes, 69.71 ± 6.19 and 45.91 ± 3.35 respectively. The ICM and TE cell number were higher in BCB+ (18.82 ± 1.77 and 50.88 ± 5.06) than BCB- (12.55 ± 1.12 and 33.36 ± 3.16 respectively). The ICM:TE ratio was not significant between BCB selected groups (1:2.70 and 1:2.65 respectively).

Before maturation, the mean diameter of BCB+ oocytes was 123.66 ± 2.72 ($\pm \text{s.e.m.}$), significantly higher ($P < 0.0001$) than BCB- (106.82 ± 2.88). After 24 h of IVM, the BCB+ group maintained their diameter while

BCB- showed a significant increase of 12 μm of the internal zona diameter (from 106.82 ± 2.88 to 118.86 ± 3.26 μm ; $P = 0.006$).

Mitochondrial activity in prepubertal sheep oocytes selected by BCB

Figure 1 shows representative images for the different mitochondrial distribution parameters. At the GV stage, 43.9% of oocytes presented homogeneous (Fig. 1B) and 56.1% showed peripheral (Fig. 1C) distribution. After maturation, 53.2% showed a homogeneous distribution while the peripheral distribution decreased up to 6.4%, the rest of the oocytes exhibited a polarised distribution (40.4%) marked by the position of active mitochondria around the metaphase spindle and polar body (Fig. 1D; $P < 0.001$). No differences were found in mitochondrial distribution between BCB+ and BCB- oocytes.

Mitochondrial activity is represented in Fig. 2 by the analysis of the fluorescence intensity in oocytes pre-labelled with the mitochondrial-specific probe. Our results indicate a relationship between mitochondrial activity, BCB oocyte status and maturation stage. Before IVM, BCB+ and BCB- oocytes showed no significant differences in mitochondrial activity between groups (2834 ± 223.42 and 3519 ± 288.48 AU respectively). After IVM, BCB- oocytes mitochondrial activity descended abruptly (from 3519 ± 288.48 to 1565 ± 113.8 AU; $P < 0.0001$) while activity in the BCB+ group did not show any changes. Between matured BCB+ and BCB- oocytes, mitochondrial activity differed significantly ($P < 0.0001$). Considering the overall oocytes and comparing mitochondrial activity at the GV and MII stages, we observed a decreasing activity during meiosis (3175 ± 253.9 to 2385 ± 233 AU $\pm \text{s.e.m.}$ respectively $P < 0.05$).

MPF activity in prepubertal sheep oocytes selected by BCB

Results in MPF activity of oocytes with different cytoplasmic quality and stage of maturation assessed by CDC2 kinase activity are presented in Fig. 3. No differences were observed in MPF activity at collection

Table 2 Embryo development of prepubertal sheep oocytes selected with different Brilliant Cresyl Blue (BCB) concentrations.

BCB concentration (μM)	Oocyte classification					
	BCB+, n (%)			BCB-, n (%)		
	Inseminated oocytes	Cleavage	Blastocyst	Inseminated oocytes	Cleavage	Blastocyst
13	107	85 (79) ^a	22 (21) ^{a,A}	204	128 (63) ^{a,b}	18 (9) ^{a,B}
26	114	77 (68) ^b	19 (17) ^{a,c,A}	192	122 (64) ^{a,b}	9 (5) ^{a,B}
39	136	90 (66) ^b	14 (10) ^{b,c}	202	145 (72) ^a	13 (6) ^a
52	123	86 (70) ^{a,b}	10 (8) ^b	207	120 (58) ^b	9 (4) ^a

Values in the same column (^{a,b,c}) or row (A, B) with different letters differ significantly (Fisher test; $P < 0.05$).

Table 3 Nuclear stage of prepubertal sheep Brilliant Cresyl Blue (BCB) selected oocytes at 17 h post-insemination.

Oocyte classification (13 µM BCB)	Oocytes at MII (%)	Inseminated oocytes	Fertilised oocytes, n (%)		
			2PN	PS	AS
Control	80	68	16 (23) ^b		2 (3)
BCB+	86	60	24 (40) ^a	3 (5)	
BCB−	72.5	64	14 (22) ^b	4 (6)	3 (5)

Different letters (^{a,b}) within a column are significantly different (Fisher test; $P<0.05$). MII, metaphase II; 2PN, one sperm tail and 2 pronuclei; PS (polyspermic), two or more sperm tails or more than 2 pronuclei; AS (asynchronous), only one pronucleus.

time in BCB+ and BCB− oocytes (0.285 ± 0.03 and 0.212 ± 0.01 optical density (OD) respectively). After IVM, MPF showed a significantly ($P<0.05$) increased activity in BCB+ with respect to BCB− oocytes (1.479 ± 0.09 and 1.184 ± 0.05 OD respectively). In both cases BCB+ and BCB− oocytes showed a significantly ($P<0.001$) increasing MPF activity from the GV to the MII stage of maturation.

Relative mRNA expression of ATP1A1, COX1, CPEB and S100A10 of BCB selected oocytes

Figure 4 shows the relative expression (RE) of the four maturation gene candidates analysed by real-time PCR in relation to BCB status and IVM. No differences in gene expression were observed in relation to BCB classification. The only difference we found was in BCB+ oocytes that showed a significantly lower amount of S100A10 transcript after IVM ($P<0.05$).

Discussion

This study was carried out to determine the best concentration of BCB stain to select competent prepubertal sheep oocytes for *in vitro* embryo production and to analyse mitochondrial distribution and activity, MPF activity and changes in the RE of ATP1A1, COX1, CPEB and S100A10 as maturation gene candidates in those BCB selected oocytes.

In cows (Pujol *et al.* 2004, Alm *et al.* 2005), goats (Rodriguez-Gonzalez *et al.* 2002) and mice (Wu *et al.* 2007) the concentration of BCB stain with the best results was 26 µM BCB for 90 min, while in pigs 13 µM BCB was sufficient to increase sperm penetration of BCB+

oocytes (Roca *et al.* 1998, Egerszegi *et al.* 2010). In our work, we decided to work with 13 µM BCB for 60 min, as it was the most specific concentration, increasing blastocyst rate from 9% (BCB−) to 21% (BCB+). We considered BCB stain not to be detrimental for oocytes because no differences were found between treatments and the control group as described by Wongsrikeao *et al.* (2006) in pigs.

Oocyte diameter is a determinant factor for completion of meiosis and acquisition of full competence for embryo development (Lonergan *et al.* 1994, Crozet *et al.* 2000). In our work, we found that the BCB test was helpful in selecting larger oocytes with 123.66 ± 2.72 µm diameter (BCB+) compared with those of 106.82 ± 2.88 µm (BCB−). This confirmed previous reports in cattle (152.6 vs 147 µm; Pujol *et al.* 2004), goats (136.6 vs 125.5 µm; Rodriguez-Gonzalez *et al.* 2002) and pigs (113.08 vs 100.29 µm; Roca *et al.* 1998) for BCB+ and BCB− respectively. After IVM, we found a significant increase of 12 µm of the internal zona diameter in BCB− oocytes, while BCB+ oocytes maintained the same diameter after 24 h of *in vitro* culture. Oocyte quality is assessed by male pronuclear formation (2PN zygotes), blastocyst yield and blastocyst cell number. In this study, we have observed significantly higher 2PN zygote and blastocyst production in BCB+ than BCB− oocytes. This increase in 2PN zygote and blastocyst development has been shown in BCB+ oocytes of buffalo (Manjunatha *et al.* 2007), cattle (Pujol *et al.* 2004, Alm *et al.* 2005, Bhojwani *et al.* 2007, Torner *et al.* 2008), goats (Rodriguez-Gonzalez *et al.* 2002), mice (Wu *et al.* 2007) and pigs (Egerszegi *et al.* 2010).

Assessment of blastocyst cell number indicates that BCB+ oocytes produce blastocysts with a higher cell number than BCB−. In prepubertal goat oocytes, Romaguera *et al.* (2010, 2011) did not find differences in blastocyst quality, assessed by blastomere ploidy and cryotolerance, between oocytes coming from different diameter follicles and between oocytes from adult and prepubertal goats, concluding that oocyte quality was positively related to blastocyst production but not to blastocyst quality. In cattle, Majerus *et al.* (2000) observed that the percentage of blastocysts obtained from calf oocytes was reduced in comparison with those from adult animals (26 vs 46%); however, the number of blastocyst cells was not different (89 and 100 respectively) and the ICM:TE ratio was similar (1:2.70 and

Table 4 Total cell numbers of *in vitro* produced blastocyst from prepubertal sheep Brilliant Cresyl Blue (BCB) selected oocytes at day 8 post-insemination.

		Cell number (mean $\pm$ S.E.M.)			Percentage/total cells (n)		
	N	Total	TE	ICM	ICM	TE	ICM:TE ratio
BCB +	24	69.71 $\pm$ 6.19 ^a	50.88 $\pm$ 5.06 ^a	18.83 $\pm$ 1.77 ^a	27.01	72.99	1:2.70
BCB −	22	45.91 $\pm$ 3.35 ^b	33.36 $\pm$ 3.16 ^b	12.55 $\pm$ 1.12 ^b	27.34	72.66	1:2.65

ICM, inner cell mass; TE, trophectoderm. Different letters (^{a,b}) within are significantly different (Student's *t*-test; $P<0.05$).

Figure 1 Representative images of active mitochondrial distribution of prepubertal sheep oocytes taken by a confocal microscope. Images were spectrally (A) coded to represent staining intensity (red is the highest intensity). Representative images of (B) homogeneous, (C) peripheral and (D) polarised mitochondrial activity distribution in lamb oocytes.

1:2.85 respectively). Selecting lamb oocytes according to the number of cumulus layers, Kelly *et al.* (2007) concluded that the percentage of day 8 blastocysts was affected by COC grade but the number of blastocyst cells was not significantly different (range 49.2–54.6 cells per blastocyst). To our knowledge, no studies on oocytes selected by BCB and embryo quality have been done. In this study, we have shown a positive relationship between BCB+ oocytes and the number of blastomeres per blastocyst.

Mitochondrial distribution and activity inside the oocyte could be a good marker of oocyte competence to develop to the blastocyst stage. The primary function of mitochondria is to generate ATP. Van Blerkom *et al.* (1995) described in human oocytes the relationship between ATP content and embryo developmental capacity where a transient decrease in ATP content can lead to embryo arrest. Therefore, these data suggest that mitochondrial activity is a determinant factor of quality and changes in mitochondrial activity can alter oocyte quality in a remarkable way. In cattle (Tarazona *et al.* 2006), horses (Torner *et al.* 2007), humans (Van Blerkom 2004) and pigs (Torner *et al.* 2004) an increase in mitochondrial activity after IVM was described. In cattle, Torner *et al.* (2008) observed higher mitochondrial activity in BCB– oocytes than in BCB+. These authors speculated that the reason for the increasing respiratory activity in low-quality oocytes was to provide ATP for still unfinished processes for cytoplasmic maturation. In pig oocytes, Egerszegi *et al.* (2010) found a higher mitochondrial activity in BCB+ compared with BCB– oocytes before IVM, but after IVM, no differences were found between either kind of oocyte. They attribute this to BCB+ oocytes increasing their respiratory activity to provide ATP for the energy-demanding processes of GVBD and the subsequent condensation of chromatin up to MII, while BCB– oocytes showed no changes in mitochondrial activity during meiosis and only a few of them reached MII stage. In our study with prepubertal sheep oocytes, we found a decrease in mitochondrial activity from the GV to the MII stage (3175 ± 253.9 – 2385 ± 233 AU $\pm$ S.E.M.). Analysing BCB+

and BCB– oocytes separately, we found that at the GV stage there were no differences in mitochondrial activity between groups. However, after IVM, BCB– oocytes showed a significant reduction in mitochondrial activity while BCB+ mitochondrial activity remained constant. This would indicate a positive relationship between mitochondrial activity at MII stage and embryo development.

Stojkovic *et al.* (2001) showed that mitochondrial reorganisation was different between morphologically good and poor quality oocytes. In our study, mitochondria migrated throughout the IVM process. Oocytes at the GV stage presented a homogeneous (43.9%) or peripheral (56.1%) mitochondrial distribution. After 24 h of IVM, MII oocytes presented a homogeneous (53.2%) distribution or mitochondria polarised around the metaphase spindle and inside the polar body (PB; 40.4%). We have previously shown (Velilla *et al.* 2006), in prepubertal goat IVM oocytes, that total mitochondria migrate from a cortical and perinuclear distribution in GV oocytes to a polarised distribution opposite the metaphase spindle and inside the PB (86%) after IVM, whereas ovulated adult goat oocytes presented a mitochondrial distribution inside the PB and aggregated to the metaphase spindle (Velilla *et al.* 2006) as we have found here in lamb oocytes. In pigs, Torner *et al.* (2004)

Figure 2 Mitochondrial activity of BCB selected oocytes assessed by fluorescence intensity before (0 h) and after IVM (24 h). Different symbols (#, *) indicate significant differences (* $P < 0.0001$; # $P < 0.05$).

Figure 3 MPF activity in BCB-exposed prepubertal sheep oocytes before (0 h) and after IVM (24 h). MPF activity is expressed as OD at 492 nm. ^{a,b,c}Indicates a significant difference in kinase activity among oocyte groups.

found that mitochondria moved from a homogeneous to a peripheral distribution; however, Brevini *et al.* (2005) showed a peripheral distribution in immature pig oocytes that became diffused after IVM. In our study, we did not find differences in mitochondrial distribution between BCB oocyte groups, while Egerszegi *et al.* (2010) concluded that, in pigs, BCB- oocytes showed more heterogeneous and non-aggregated mitochondrial distribution than BCB+ oocytes. Different authors have reported differences in mitochondrial distribution patterns among species, during IVM and *in vivo* maturation (Stojkovic *et al.* 2001, Sun *et al.* 2001, Torner *et al.* 2004, Velilla *et al.* 2006, Torner *et al.* 2007, Dell'Aquila *et al.* 2009, Egerszegi *et al.* 2010).

Previous studies showed that GV-oocytes do not present MPF activity (Dedieu *et al.* 1996) but, in our study, we detected MPF activity before IVM; this may be due to the BCB staining time. We presume that oocytes could restart meiosis and so they were in GVBD instead of GV when MPF analysis took place. After IVM, we observed significantly higher MPF activity in BCB+ oocytes than in BCB- oocytes. Salamone *et al.* (2001) reported that calf oocytes undergo cleavage and blastocyst production at significantly lower rates than cow oocytes and this was correlated to a lower activity of MPF in these oocytes. Closer to our study, comparing prepubertal and adult sheep oocytes, Ledda *et al.* (2001) showed that the low competence in prepubertal oocytes could be due to morphological anomalies and alterations in physiological activity due to the evidence of low MPF activity after IVM. Bogliolo *et al.* (2004) showed a higher MPF activity in *in vivo* matured oocytes than in *in vitro* matured cat oocytes, suggesting a possibly incomplete cytoplasmic maturation after culture. High MPF activity was observed by Anguita *et al.* (2007) in oocytes with larger diameter and better competence to develop to the blastocyst stage. In addition, MPF activity has also been related to an increase in developmental competence of oocytes treated with caffeine during nuclear transfer (Kawahara *et al.* 2005). In relation to mitochondrial and MPF activities analysed in this work, we could speculate that there is a positive relationship between the ATP produced by the active mitochondria and the ATP production needed to phosphorylate p34^{cdc2} and activate the MPF complex.

The genes analysed in this work were related to metabolism (*ATP1A1* and *COX1*) and constitutive function of the cell (*CPEB* and *S100A10*). Oocytes selected by BCB did not show any differences in RE in any of the studied genes, in spite of the higher embryo development observed in BCB+ oocytes. However, in bovine oocytes, Opiela *et al.* (2010) found higher RE of the *COX1* gene in immature BCB+ with respect to BCB- and Torner *et al.* (2008) showed a higher RE of *S100A10* in matured BCB- than BCB+ oocytes. During IVM in prepubertal sheep oocytes, we found a significant decreasing mRNA RE in *S100A10* in BCB+ oocytes.

In conclusion, exposing prepubertal sheep oocytes to 13 µM BCB for 60 min stains the largest and most competent oocytes to develop to the blastocyst stage. After IVM, the more competent oocytes (BCB+) presented higher mitochondrial and MPF activity with respect to BCB- oocytes. BCB+ oocytes produced blastocysts with higher numbers of cells than BCB- oocytes. Mitochondrial distribution and mRNA expression of *ATP1A1*, *COX1*, *CPEB* and *S100A10* were not affected by oocyte quality.

Materials and Methods

Chemical

All chemicals were purchased from Sigma-Aldrich Chemical Co. unless otherwise specified.

Oocyte collection

Ovaries from prepubertal ewes (3–6 months old), were obtained from a local abattoir and transported to laboratory in sterile dulbecco's (PBS) held at 34–37 °C and were washed

Figure 4 Relative mRNA expression of *S100A10*, *CPEB*, *COX1* and *ATP1A1* transcripts before (0 h) and after IVM (24 h) of prepubertal sheep BCB selected oocytes. Different letters (a, b) indicate significant differences ($P < 0.05$; one-way ANOVA with Tukey's multiple comparison post-test to compare the four groups of each gene).

in PBS containing antibiotic–antimycotic (AB, Gibco cat no. 14240-062). COCs were recovered by slicing the surface of the ovary in HEPES-buffered TCM-199 medium with 0.5 g/l fraction V BSA. Oocytes with two or more complete layers of compact cumulus cells and homogeneous cytoplasm were used.

BCB test

Immediately after collection, COCs were washed three times in mPBS (PBS supplemented with 1 g/l glucose, 36 mg/l sodium pyruvate, 0.5 g/l BSA and 0.05 g/l gentamicin) and exposed to different concentrations of BCB (13, 26, 39 and 52 μ M) diluted in mPBS for 1 h at 38.5 °C in a humidified air atmosphere. After BCB incubation, oocytes were washed three times in mPBS and classified into two groups depending on their cytoplasm colouration: oocytes with blue cytoplasm or hypothetically grown oocytes (BCB+) and oocytes without blue cytoplasm colouration or hypothetically growing oocytes (BCB–).

IVM of oocytes

Groups of 35–40 COCs of BCB+, BCB– and control (oocytes not exposed to BCB stain), were washed and cultured for 24 h at 38.5 °C in a humidified air atmosphere with 5% CO₂ in 500 μ l of maturation medium covered with mineral oil. Maturation medium consisted of TCM-199 supplemented with 5 μ g/ml LH, 5 μ g/ml FSH, 1 μ g/ml 17 β oestradiol, 10 ng/ml epidermal growth factor, 0.2 mM sodium pyruvate, 2 mM glutamine, 100 μ M cysteamine, 10% (v/v) fetal bovine serum (FBS) and 2% (v/v) AB.

IVF and embryo culture

Fresh semen, obtained from three rams of proven fertility, was kept at room temperature (25 °C) for 90 min (Ptak *et al.* 1999). Highly motile spermatozoa were selected by Ovipure density gradient (Nidacon EVB S.L., Barcelona, Spain) and fertilised with 1×10^6 spermatozoa/ml. Matured oocytes were partially denuded by gentle pipetting and transferred into fertilisation medium consisting of synthetic oviductal fluid (SOF; Holm *et al.* 1999). Fertilisation was carried out in drops of 50 μ l of SOF medium supplemented with 10% of oestrous sheep serum with a maximum of 15 oocytes per drop and incubated for 20 h at 38.5 °C, 5% CO₂ and 5% O₂ in a humidified atmosphere.

After IVF, presumptive zygotes were completely denuded with gently pipetting and cultured in groups of six zygotes for 8 days in 20 μ l culture drops consisting of SOF medium supplemented with 10% (v/v) FBS under the same atmospheric conditions.

Blastocyst differential staining

Blastocyst differential stain protocol was taken and modified from Thouas (2001). Briefly, 8-day-old blastocysts were first incubated for ~15 s or until TE visibly changed colour in

solution 1 (PBS–BSA free with 1% Triton X-100 and 100 μ g/ml propidium iodide). Immediately afterwards they were transferred to solution 2 (100% ethanol with 25 μ g/ml Hoechst 33258) for 1 h.

Stained blastocysts were transferred from solution 2 directly to a glass with a drop of glycerol, taking care to avoid carrying over an excessive amount of solution, flattened with a coverslip and visualised under a microscope for cell counting. We used an Olympus BX50 with a u.v. lamp and excitation filter of 460 nm for blue and red fluorescence.

The intense pink colour represents the chromatin in nuclei of permeabilised TE cells that are stained both red (propidium iodide) and blue (Hoechst). ICM nuclei remain blue, because these cells have not been permeabilised.

Oocyte diameter and nuclear stage of 13 μ M BCB selected oocytes

Oocyte diameter was measured after selection by the BCB test (0 h) and after IVM (24 h). Oocytes were denuded and fixed with 3% paraformaldehyde and mounted on poly-L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring and then covered with a drop of Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA). Oocytes were measured (inside zona pellucida) by taking a picture under the microscope and analysed with MetaMorph imaging software (MetaMorph 6.2.6 Software, Universal Imaging Corporation).

To evaluate the nuclear and pronuclear stages, matured and fertilised (after 17 h of IVF) COCs were denuded as described before and fixed during 24 h in ethanol: acetic (3:1) and stained it with 1% lacmoid. Oocytes were considered correctly matured when they reached the MII stage and correctly fertilised when one sperm tail and 2 pronuclei (2PN) were visible, polyspermic when two or more sperm tails or more than 2 pronuclei (PS) were visible and asynchronous when only one pronucleus and a non-decondensed sperm were present.

Mitochondrial activity of BCB selected oocytes

Prepubertal sheep oocytes selected with 13 μ M BCB at 0 and 24 h post IVM were totally denuded with gentle pipetting and incubated in mPBS with 3% (v/v) BSA containing 200 nM MitoTracker Orange CMTMRos (Molecular Probes, Inc., Eugene, OR, USA) under culture conditions for 60 min. The probe is readily sequestered only by active organelles depending on their oxidative activity. Immediately after staining, oocytes were washed three times in mPBS and fixed for 60 min at 38 °C in 3% paraformaldehyde. After fixation, oocytes were properly washed in PBS 0.1% (v/v) BSA and stained for 5 min in 1 μ g/ml Hoechst 33342 solution. Finally, groups of ten oocytes were mounted on poly-L-lysine-treated coverslips fitted with a self-adhesive reinforcement ring and covered with a drop of Vectashield mounting medium. Slides were then sealed with nail varnish and stored at –20 °C protected from light for 6 days until their analysis under a confocal microscope.

Confocal analysis

A laser scanning confocal microscope (Spectral Leica TCS-SP5, Mannheim, Germany) was used to examine active mitochondria (MitoTracker Orange CMTMRos, excitation 554 nm) and chromatin (Hoechst; excitation 405 nm). The mitochondrial distribution pattern (representative images in Fig. 1) was characterised with 60× magnification under mineral oil and classified as homogeneous (fluorescence throughout the cytoplasm, Fig. 1B), peripheral (fluorescence in the cortex, Fig. 1C) or polarised (fluorescence near the MII spindle, Fig. 1D) according to mitochondrial distribution.

For mitochondrial intensity the microscope objective, pinhole, filters, offset, gain, pixels and laser potency were kept constant throughout the experiment. Twenty serial cuts of 1 µm each in the region of major intensity were performed. The 20 images of each oocyte were added in a unique picture and analysed with MetaMorph imaging software. To express the fluorescence intensity, we used arbitrary unit of the mean fluorescence intensity, which is defined as the sum of fluorescence intensity in greyscale of every pixel in the cytoplasm of the oocyte divided by the sum of all pixels of the same region.

MPF activity of BCB selected oocytes

Before and after IVM, groups of 20 oocytes BCB+, BCB– (three replicates) were washed three times in PBS, and placed in tubes containing 5 µl of lysis buffer (50 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 5 mM EDTA, 0.01% Brij35, 1 mM PMSF, 0.05 mg/ml leupeptin, 50 mM 2-mercaptoethanol, 25 mM β-glycerophosphate and 1 mM Na-orthovanadate). The samples were frozen in liquid nitrogen and sonicated three times at 1 °C for 25 s. Cell extracts were stored at –80 °C until use.

CDC2 assay was performed by the MESACUP CDC2 kinase assay kit (MBL, Madrid, Spain) following the manufacturer's protocol. Oocyte extracts (5 µl) were mixed with 10× CDC2 Reaction Buffer (25 mM Hepes buffer pH 7.5 and 10 mM MgCl₂) and 10% biotinylated MV Peptide (SLYSSPGAYC). The phosphorylation reaction was started adding 0.1 mM ATP (Sigma–Aldrich), in a final volume of 50 µl. The mixture was incubated at 30 °C for 30 min. The reaction was finished by

adding 200 µl of phosphorylation Stop Reagent (PBS containing 50 mM EGTA). The phosphorylated MV peptide was detected by ELISA at 492 nm and expressed as OD.

Real-time PCR quantification of ATP1A1, COX1, CPEB and S100A10 of BCB selected oocytes

Groups of 15 denuded prepubertal sheep oocytes (four replicates) stained with 13 µM BCB were taken at 0 and 24 h post IVM and stored at –80 °C in 100 µl Trizol (Invitrogen) until use. For RNA extraction, the addition of a known amount of luciferase (0.2 pg/µl, Promega) was added as an exogenous standard. To avoid contamination with genomic DNA, total RNA preparations were incubated during 10 min with RQ1 DNase (Promega) as described in the manufacturer's protocol. Reverse transcription was performed by extended cDNA using Oligo (dT) 15 primers during 5 min at 70 °C and 1 h at 65 °C using superscript III (200 U/µM; Invitrogen). Relative qualitative PCR analysis was performed in MyiQ apparatus (Bio-Rad Laboratories). Samples were distributed in the plate by a robotic distributor (Eppendorf) and reactions were performed in duplicate using SYBR Green Fluorophore kit (Bio-Rad). Reactions were performed in 20 µl final volume (in duplicate) and PCR cycling conditions were 95 °C for 3 min followed by 40 cycles of denaturing (30 s, 95 °C), annealing (30 s, 60 °C) and elongation (20 s, 72 °C). The specificity of each PCR product was determined by a melting curve analysis and the amplicon size determination in agarose gels. For each gene, a standard curve was included, consisting of corresponding plasmid DNA fragments from 1 pg to 0.1 fg, purified with QIAquick PCR Purification Kit (Qiagen). Correlation coefficients and PCR efficiencies were considered between 85 and 100%. Primer sequences are listed in Table 5. The results for mRNA were normalised according to the relative concentration of the internal standard, luciferase and 18S.

Statistical analysis

Analysis among treatments (BCB stain, IVM and IVF) were performed by Fisher's exact test. Blastocyst cell numbers were analysed by Student's *t*-test. Gene and MPF analysis was performed by one-way ANOVA in GraphPad Prism v 3 (GraphPad Software, San Diego, CA, USA).

Table 5 Oligonucleotide primer sequences used in this study.

Gene	Primer	Sequence 5'–3'	GenBank accession no.	Product size (bp)
S100A10	Sense	CCGCCAAGGTTTCAACAGACTTC	EE822394	271
	Antisense	ATGGTGAGCCCAGCGATTAGC		
CPEB	Sense	CCTCCCAGATGCAAATGACT	DY514003	235
	Antisense	CTTAATGGAGGGTGCTGGAA		
COX1	Sense	TGGAGGACAATATCAAGGGAGGAG	CO000988	471
	Antisense	GGACCGAAACCTGAACACAACC		
ATP1A1	Sense	GAACGGCTTCCTCCCTAATC	NM_001009360	207
	Antisense	ACGGAATTCCTCCTGGTCTT		
LUCI	Sense	TCATTCTCGCCAAAAGCACTC		140
	Antisense	AGCCCATATCCTTGTCGTATCC		
18S	Sense	AGAAACGGCTACCATCCAA	DQ222453.1	90
	Antisense	CCTGTATTGTTATTTTCCT		

The individual mitochondrial intensity (arbitrary unit) data were analysed as log10 to normalise them. Statistical analysis was conducted by PROC MIXED (with number of treatments as random parameter) of SAS (version 9.2 Inst., Inc., Cary, NC, USA) according to Tukey's multiple comparison test. The statistical model contained the fixed effects of BCB (+ versus -) and maturation state (0 vs 24 h) and first-order interaction between BCB and maturation state and residual error. Differences were declared significant at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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