Oocyte quality

Assessment of prepubertal sheep oocyte competence for *in vitro* embryo production by the *Brilliant Cresyl Blue* test.

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CERTIFICA:

Que la tesis titulada "**Assessment of prepubertal sheep oocyte competence for *in vitro* embryo production by the *Brilliant Cresyl Blue* test.**" presentada por María Gracia Catalá para optar al grado de Doctor, se realizó bajo mi dirección y con un financiamiento del Ministerio de Ciencia e Innovación (AGL 2007 60227/GAN y AGL2011-23784) y una beca otorgada por la Universidad Autónoma de Barcelona (UAB-2007FI00193).

I para que conste a los efectos oportunos, firmo la presente en Bellaterra (Cerdanyola del Vallès), el 12 de marzo de 2012

Dra. Maria-Teresa Paramio Nieto

A mis padres,

A mi hermano,

A mi hermana del alma,

A Amine,

C*hapter 1:*

General background

Assisted reproductive technologies (ART) have been one of the major tools towards increasing productivity in livestock industry. In this field, artificial insemination (AI) is the earliest and most powerful among the reproductive technologies because it is easy to perform, cost-effective, and highly successful (Vishwanath. 2003). For over the years AI has been used to obtain offspring from genetically superior and from sub-fertile animals. Moreover, by the 1960´s, significant improvements in cryopreservation and storage of semen made AI even more accessible to livestock producers (Vishwanath. 2003). In the modern dairy industry, new ART techniques were developed as cryopreservation not only of semen but also of gametes and embryos, induction of multiple ovulations, embryo transfer, *in vitro* fertilization (IVF), sex determination of sperm or embryos, nuclear transfer, cloning, etc. In the small ruminants as sheep and goat, these techniques had a lower development.

Despite the improvement in ART protocols, the pregnancy rates are still relatively low and early embryonic mortality has been reported after embryo transfer. It has been shown that 40% of total embryonic losses occur between day 8 and day 17 of pregnancy (Thatcher et al. 1994) and one of the reasons could be due to the oocyte quality (Snijders et al. 2000). The oocyte quality is used as synonymous of oocyte competence, defined as the ability of an oocyte to resume meiosis, cleave following fertilization, develop to the blastocyst stage, induce a pregnancy and bring the offspring to term in good health (Sirard et al. 2006). The oocyte competence is acquired gradually during the course of folliculogenesis as the oocyte grows and its somatic cells cohort differentiates (Eppig et al. 1994). Many factors have been shown to affect the oocyte competence as: follicle size (Lonergan et al. 1994; Romaguera et al. 2011), phase of follicular wave (Machatkovaa et al. 2004), hormonal stimulation (Sirard et al. 2006), maturation environment (Warzych et al. 2007), season (Sartori et al. 2002), nutrition (Fouladi-Nashta et al. 2007) and donor's age (Rizos et al. 2005).

The assessment of oocyte quality is one of the major objectives in ART, especially in human where the use of the best quality oocytes to be inseminated may improve significantly the embryo production. Consequently, multiple methods of oocyte selection have been proposed. One of the most popular is to select the oocyte by morphology which is relatively quick and simple; however this simple technique leads to identify more frequently the negative than the positive aspects, and overall it is not fully satisfactory (Balaban and Urman. 2006). Other methods have been proposed as the use of polarizing microscopy analysis (Heindryckx et al. 2011), gene expression of the granulosa cells or in the oocyte itself (Patrizio et al. 2007), polar body biopsy to screen oocytes with chromosomal defects deriving from errors in the meiotic division (Dawson et al. 2006). Most of these techniques are quite complicated, require

expensive laboratory equipment and time-consuming procedures, and some of them are invasive and consequently are not currently applicable in the clinical practice.

In research, the law is extremely restrictive in the use of human embryos; consequently using animals has become through time a valuable tool. The bovine is the mostly used ruminant in ART research because of its economic importance and its wide geographical distribution, although using small ruminants as oocyte donor could be an interesting alternative. Using the sheep (*Ovis aries*) can bring great benefits to reproduction research because of their younger age to reach puberty, short gestational periods, the possibility to have more than one offspring in a single gestation and the maintenance cost are lower than in bovine. Moreover, sheep have been domesticated for over 10,000 years and are also widespread across the world, being adapted to many different climatic conditions. During the past 60 years, sheep have been the subject of considerable research starting in 1949 with an experimental super ovulation protocol (Ortavant et al. 1949) until nowadays in which sheep receptors, genes (Leoni et al. 2007; Kyasari et al. 2012) and proteins (Grazul-Bilska et al. 2011) are being studied. In addition, using prepubertal animals as oocyte donor has some additional benefits comparing to the use of adult donors, as for example; reducing the generational interval (Duby et al. 1996), their ovaries produce a major number of oocytes than adult (Koeman et al. 2003) and this oocytes could serve as a model of low quality oocyte in research as they are characterized of having an abnormal cytoplasmic maturation and lower ability to achieve the blastocyst stage (Revel et al. 1995; O'Brien et al. 1996; Armstrong. 2001). One of the difficulties of using ovaries coming from prepubertal animals, is to release the complex oocyte cumulus (COCs) by traditional follicular aspiration because of having a high percentage of antral follicles with a smaller diameter than 3 mm (Martino et al. 1994). Consequently, the release of these oocytes is made by slicing the ovary surface obtaining oocytes with heterogeneous diameter, different COC morphology and stage of atresia.

The Brilliant Cresyl Blue (BCB) test has been successfully used as a non invasive methodology to select oocytes with a higher diameter and more competent to develop up to the blastocyst stage in cow (Pujol et al. 2004; Alm et al. 2005; Bhojwani et al. 2007; Torner et al. 2008; Opiela et al. 2010), pig (Ericsson et al. 1993; Roca et al. 1998; El Shourbagy et al. 2006; Egerszegi et al. 2010), goats (Rodriguez-Gonzalez et al. 2002; Rodriguez-Gonzalez et al. 2003; Kątska-Książkiewicz et al. 2007), mouse (Wu et al. 2007) and buffalo (Manjunatha et al. 2007). To our knowledge there are no previous reports using this stain to select oocytes by their competence in the sheep.

The aim of this study is to test the ability of the BCB staining to select the more competent sheep oocytes for in vitro embryo production. Also, in this work we pretend to improve the

knowledge about oocyte competence related to their cytoplasmic and molecular performances, their responses to different techniques of fertilization and the in vitro culture media needed to improve the blastocyst production.

Chapter 2.

Bibliographical Revision

2.1. *Current situation of the in vitro embryo production (IVEP) in sheep*

2.1.1. *In vitro* maturation (IVM)

A correct maturation of the cumulus-oocyte complex (COC) is one of the most important factors which determine the entry of the oocyte into metaphase II (MII), subsequent successful fertilization, as well as the ability of an embryo to undergo an appropriate growth and development. Performing the COC maturation under *in vitro* conditions provides an excellent opportunity for having cheap and abundant oocytes for carrying out basic research and for the application of emerging biotechnologies like cloning and transgenesis.

Several aspects of the IVM of sheep oocytes have been studied (Wani et al. 2000; Rao et al. 2002). Sheep COC's are most commonly matured in Tissue Culture Medium (TCM199) containing Earle's salts. The supplementation of the IVM medium with epidermal growth factor (EGF) (Guler et al. 2000), mare serum (Motlagh et al. 2008), fetal calf serum (FCS) (Ghasemzadeh Nava and Tajik. 2000), estrous sheep serum (ESS) (Ghasemzadeh Nava and Tajik. 2000), insulin –like growth factor (IGF-I) (Guler et al. 2000) and cysteamine (de Matos et al. 2002) among others stimulate sheep oocyte nuclear and cytoplasmic maturation. After the improvements in the IVM media, currently the most commonly media used to *in vitro* mature sheep oocytes is the TCM199 supplemented 2 mM glutamine, 100 μM cysteamine, 0.3 mM sodium pyruvate, 10% fetal bovine serum (FBS), 5 μg/mL FSH, 5 μg/mL LH and 1 μg/mL estradiol (Loi et al. 2008). Moreover supplementing the IVM media with ESS instead of FBS has also shown good results (Bebbere et al. 2010).

2.1.2. IVF and sperm capacitation.

The first reports of IVF in sheep used heparin to capacitate the ram spermatozoa reaching an 80% of fertilization but only 15% were viable embryos (Slavik et al. 1992). However, the use of ESS instead of heparin rapidly gained importance by showing a fertilization rate of 85% (Huneau et al. 1994) and 56% reached the blastocyst stage (Walker et al. 1994). The ESS works through the binding-protein albumin that may facilitate the sperm capacitation by contributing to the depletion of the sperm cholesterol membrane (Huneau et al. 1994). Ccurrently most of the authors use the ESS to capacitate fresh and frozen semen using a concentration of 2 to 20% of ESS (Walker et al. 1994). Bebbere *et al* (2010) using frozen ram semen capacitated with 2% ESS reached 54% of blastocyst using adult sheep oocytes and Shirazi (2009) with fresh semen and 20% ESS reached a 34% of blastocyst.

2.1.3. Intracytoplasmic Sperm Injection

Intracytoplasmic sperm injection (ICSI) consists in fertilizing a MII oocyte by the injection of a single spermatozoon into the cytoplasm with both the acrosome and sperm membrane intact. This technique was reported with success for the first time in hamsters 30 years ago (Uehara and Yanagimachi. 1976). Since then, the ICSI has become the most commonly used procedure to overcome male infertility problems in human reproduction. In animals, the ICSI has been in general used for research purposes, in which studies with sex-sorted semen (Wilson et al. 2006), sperm mediated gene transfer (Lavitrano et al. 2006; Pereyra-Bonnet et al. 2011) and cryopreserved oocytes (Matson et al. 1997; Pope et al. 2012) are the most commonly reported.

The first lamb born after oocyte maturation, sperm sexing by flow cytometry and ICSI was published by Catt et al. (1996). For the ICSI procedure they have directly injected the spermatozoa into the cytoplasm without any chemical activation obtaining a low efficiency of the technique that was traduced in 251 oocytes injected and transferred but only one lamb arrived to term. Later, this same group reported that the manipulation by itself was not enough to cause proper oocyte activation and that the addition of calcium in the culture media increased the efficiency of the technique (Gomez et al. 1998). More recently, Shirazi et al. (2009) tried to determine the need of an activation protocol after sheep sperm injection, concluding that the chemical activation of oocytes must be considered as an essential part of ICSI in this specie.

2.1.4. Parthenogenetic activation

After the entry of the sperm, mammalian oocytes exhibit an increase of the intracellular calcium induced by the same sperm. These transient calcium peaks are propagated throughout the fertilized oocyte in the form of a wave and initiate both the cortical granule exocytosis and escape from the MII arrest to become a zygote [revised by (Loi et al. 1998; Nakada and Mizuno. 1998)].

Oocyte activation protocols have been developed to induce artificially the intracellular calcium levels in the oocyte cytoplasm. This is achieved by exposing the oocyte to a calcium ionomicyn or ionophore and subsequently culturing it with a persistent kinase inhibitor such as 6-DMAP (6-dimethyl amino purine). The treatment with ionomycin

alone caused the resumption of meiosis but no pronuclear formation and the 6-DMAP alone did not cause any resumption of meiosis or pronuclear formation. So, it is important the combination of the two compounds to reach the pronuclear stage (Susko-Parrish et al. 1994).

In sheep, Alexander *et al.* (2006) using the combination of these two compounds produced 21 % of blastocysts; he also showed that using cycloheximide instead of the 6-DMAP it is also possible to produce blastocyst but in a lower percentage (15%). Loi *et al.* (1998), using the combination of ionomicyn and 6-DMAP to activate sheep nuclear transfer oocytes, reached an efficiency of 83% of blastocyst compared to 25% with no activation protocol.

2.1.5. Embryo culture and blastocyst production.

The most common media used during *in vitro* culture (IVC) of embryos is the Synthetical Oviductal Fluid (SOF: (Tervit et al. 1972). From the beginning, this media showed good results in culturing embryos under *in vitro* conditions obtaining 25 lambs born after 6 days of IVC (Tervit and Rowson. 1974). In addition, supplementing the SOF media with serum (20% vs. 40%) (Thompson et al. 1998) and BSA (18% vs. 28%) (Carolan et al. 1995) increased blastocyst percentage significantly. Furthermore, the addition of amino acids (aa) to this media appears to be beneficial in sheep producing 58% of blastocyst versus 22% when the aa were not added to the media (Walker et al. 1996).

2.2. *Study of the oocyte*

2.2.1. Meiosis: nuclear and cytoplasmic maturation.

In mammals, oocytes are arrested for several weeks, months or years in prophase of the first meiotic division. During this long period, oocytes accumulate molecules of mRNA, proteins, lipids and sugars as well as they gradually increase in size. The accumulation of all necessary sources of energy and information during oocyte growth is essential for the final step of oogenesis: the oocyte maturation.

Maturation consists of two interlinked and mutually dependent processes: cytoplasmic and nuclear maturation. The cytoplasmic maturation of the oocyte includes cytoplasmic changes as organelle redistribution, micro and macro molecular changes that occur during oocyte maturation. These modifications contribute to the oocyte's ability to undergo: nuclear

maturation, successful fertilization, cleavage and the development at least until the activation of the embryonic genome.

Nuclear maturation includes chromatin changes during the oocyte maturation starting from germinal vesicle (GV) breakdown (GVBD) through Meiosis I and Meiosis II when the oocyte is finally arrested in the MII stage. At this moment the oocyte is physiologically prepared to complete the second meiotic division upon fertilization. Under *in vivo* conditions, only fully grown oocytes can resume meiosis which implies that cytoplasmic changes that occur before maturation are essential for the acquisition of the developmental competence [Revised by Marteil et al (2009)]. However, when oocytes are removed manually before ovulation from an antral follicle, the separation triggers a pseudo-maturation event leading in general to the completion of the first meiotic division and the arrest at the MII stage. This process has been called spontaneous maturation and is believed to be induced by the removal of the oocyte maturation inhibitor (OMI) present in the follicle where cAMP is involved [Revised by Sirard (2011)]. A comparison between oocytes that were removed from the follicular environment and *in vitro* matured compared to *in vivo* matured oocytes, showed the same rates of nuclear maturation, fertilization and cleavage, but the percentage of blastocyst was significantly lower on *in vitro* matured group [30% vs. 60%, revised by (Sirard and Blondin. 1996)] indicating that the cytoplasmic competence must be different between the *in vitro* and the *in vivo* matured oocytes.

2.2.3. Maturation Promoting Factor.

Meiosis is regulated by the maturation-promoting factor (MPF). This universal cell cycle regulator is a heterodimer protein composed of two subunits, the catalytic subunit p34cdc2 (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 phosphorilation of p34cdc2 on threonine 161 by the protein kinase CAK (Cdc2 activation kinase) and dephosphorylation on threonine 14 and tyrosine 15 by Cdc25 phosphate is necessary.

The MPF activity appears just before GVBD increasing until metaphase I (MI); its activity decreases in the anaphase-telophase while its maximum level is reached at the MII stage (Figure 1). Incompetent goat oocytes have a limited amount of Cyclin B1 (Hue et al. 1997) and p34cdc2 (Anguita et al. 2007). In calf and lamb oocytes the MPF activity is significantly lower than in cow and ewe oocytes (Ledda et al. 2001; Salamone et al. 2001) whereas Han D *et al.* (2010), showed that the MPF activity of prepubertal mice oocytes was significantly higher than adult mice oocytes, suggesting a difference in the mechanisms according to species. In prepubertal

goats, Anguita *et al*. (2007) showed higher MPF activity and competence in oocytes with a diameter larger than 135 µm compared to the smaller diameters.

Figure 1: Schematic representation of MPF activity during oocyte maturation.
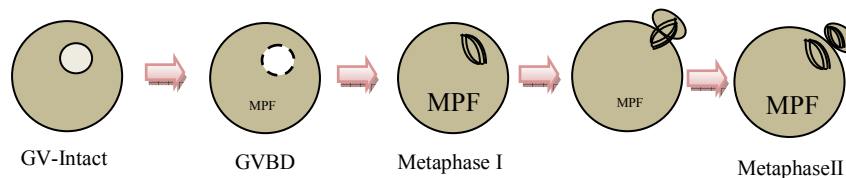
2.2.4. Mitochondria and ATP

Mitochondria are maternally inherited organelles that use oxidative phosphorylation to supply energy as adenosine triphosphate (ATP) to the cell (Stojkovic et al. 2001). This source of ATP, has a central role in the establishment of the developmental competence (Van Blerkom. 2004; Van Blerkom et al. 2008). Even though mitochondria are the most abundant organelles in the oocyte, little is known about their different functions.

The mitochondria distribution and activity change during oocyte maturation and fertilization with the aim of bringing mitochondria to the region of the cell where a higher level of ATP (Van Blerkom and Runner. 1984) or calcium (Sousa et al. 1997) are required. Energy in the form of ATP is crucial; spindle formation and chromosome behavior depend on the expression and activity of motor proteins, which use ATP as their energy source. It has been proposed that mitochondria reorganization and ATP levels are influenced by the oocyte quality (Stojkovic et al. 2001), compactness of the cumulus (Torner et al. 2007) and cumulus apoptosis (Torner et al. 2004), GnRH (Dell'Aquila et al. 2009) and the microtubule cytoplasmic network (Brevini et al. 2005) affecting the early stages of the embryo (Tarazona et al. 2006). Therefore, several authors concluded that better quality oocytes contained significantly higher ATP levels and produced significantly higher blastocyst rates after fertilization (Van Blerkom et al. 1995; Stojkovic et al. 2001; Van Blerkom. 2004).

2.2.5. Gene expression

In the last few years, the study of mammalian genes has been the focus of several studies in the belief that a good expression pattern could derive in a successful oogenesis, folliculogenesis, fertilization and early embryonic development. In the course of acquiring the oocyte competencies and a good embryo development a correct mRNA transcription is a crucial process occurring in the cytoplasm (Crozet et al. 1981; Brevini-Gandolfi and Gandolfi. 2001;

Patel et al. 2007).The mRNA content in oocytes is affected by animal nutrition (Pisani et al. 2008), donor age (Hamatani et al. 2004), follicle diameter (Caixeta et al. 2009), IVM culture media (Saadeldin et al. 2011; Salhab et al. 2011), in vivo and in vitro conditions (Wells and Patrizio. 2008), apoptosis (Li et al. 2009) and the cumulus cells (Adriaenssens et al. 2010) among others.

In this thesis we are going to study the expression of four genes in relation with the oocyte quality; two genes involved in metabolism: *ATP1A1* (ATPase NaC/KC transporting a 1) and *COX1* (cytochrome c oxidase subunit 1), and two genes involved in the constitutive function of the cell: *CPEB* (cytoplasmic polyadenylation-element-binding protein) and *S100A10* (calcium-binding protein).

ATP1A1 gene is translated in an enzyme responsible for the transport of Na+ out of and K+ into the cell and that is an important key regulator of bovine blastocyst formation and is necessary for the in vitro production of healthy bovine embryos (Watson et al. 1999). *CPEB* plays an important role in the regulation of the mRNA translation targets required for oocyte maturation (Cai et al. 2010). *COX1* is a gene that produces a mitochondrial energy-transfer enzyme of the respiratory chain. Opiela et al. (2010) found a high expression of *COX1* in oocytes of better quality versus lower quality oocytes. According to Tingaud-Sequeira et al. (2009), *S100A10* plays an antiapoptotic role and that a high expression levels of *S100A10* in the follicles may have a dual function protecting follicular cells from apoptosis during atresia and acting as chemoattractant for leukocytes and macrophages. After a microarrays of bovine oocytes, Torner *et al*. (2008) showed a higher expression of *S100A10* in lower quality than in high quality oocytes.

2.2.6. Noninvasive oocyte quality assessment: *Brilliant Cresyl Blue* Stain.

With the aim of selecting the most competent oocytes, relevant results in goat (Rodriguez-Gonzalez et al. 2002), bovine (Pujol et al. 2004; Alm et al. 2005; Bhojwani et al. 2007; Torner et al. 2008; Opiela et al. 2010), pig (El Shourbagy et al. 2006), mouse (Wu et al. 2007) and buffalo (Manjunatha et al. 2007) were published when the Brilliant Cresyl Blue (BCB) stain was used to select oocytes prior to the IVM. This is a non invasive methodology that allows selecting oocytes with larger diameters among a heterogeneous pool. Brilliant Cresyl Blue is a compound (Figure 2; $C_{17}H_{20}N_3OCl \cdot 1/2ZnCl_2$) with a molecular weight of 385.96 g/mol which is used to determine the intracellular activity of glucose-6-phosphate dehydrogenase (G6PDH). The G6PDH is a regulatory enzyme synthesized within the oocyte during oogenesis, and is a component of the pentose phosphate pathway (Figure 3) that controls the flow of carbon through this pathway and produces reducing equivalents in the form of NADPH to meet

cellular needs for reductive biosynthesis and maintenance of the cellular redox state. The activity of the G6PDH gradually decreases as oocyte reach their growth phase (Mangia and Epstein. 1975). The BCB dye can be reduced by the G6PDH enzyme activity, thus the oocytes that have reached their growth phase cannot reduce BCB to a colorless compound exhibiting a blue colored cytoplasm (BCB+). However, the growing oocytes are expected to have a high activity of G6PDH and be able to reduce the blue compound which results in a colorless oocyte cytoplasm (BCB–).

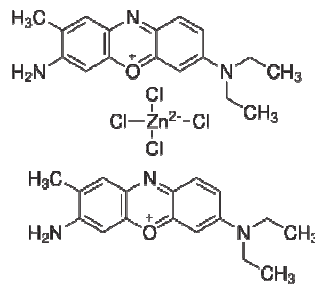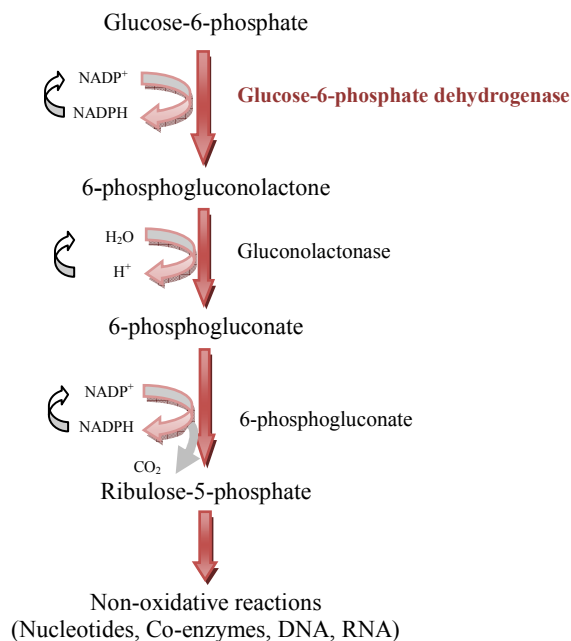Figure 2: Brilliant Cresyl Blue compound

Figure 3: A reduce scheme of the Pentose Phosphate Pathway

The first studies using the BCB compound appeared in the 80's, when this stain was used as a colorimetric assay to detect X-linked enzymes such as G6PDH to predict the sex of the embryos, resulting in a 64% of efficiency in sex prediction (Williams. 1986). Some years later, Ericsson *et al.* (1993) changed the use of the BCB stain in predicting sex, and used it to select more competent porcine oocytes for IVM and IVF. He reported for the first time a significant increase in maturation (82% vs. 49%) and sperm penetration (51% vs 26%) for the BCB+ respect the BCB- oocytes.

In our laboratory we have previously shown the usefulness of the BCB stain to select the larger and more competent oocytes for *in vitro* blastocyst production. The percentages of total embryos undergoing development to the morula plus blastocyst stages were higher in the group of BCB+ than in BCB− oocytes in prepubertal goat [12% vs. 4%, respectively; (Rodriguez-Gonzalez et al. 2002)] and in heifers [13% vs. 2%, respectively; (Pujol et al. 2004)]. Additional reports reiterate the value of the BCB stain for the selection of developmentally competent oocytes related to bovine nuclear transfer procedures (Bhojwani et al. 2007) and in COC's collected by OPU (Tagawa et al. 2006). Torner *et al.* (2008) studied in bovine the efficiency of the BCB stain by analyzing the phosphorylation pattern of protein kinases, the cDNA microarray for gene expression profiles, the fluorescence labeling and photometric measurement for chromatin configuration of the nucleus and the mitochondrial activity of the oocytes. They reported a higher phosphorylation levels of Akt and MAP kinases and an increase in transcription of genes that are involved in regulating transcription (SMARCA5), cell cycle (nuclear autoantigenic sperm protein, NASP) and protein biosynthesis (RPS274A and EF1A) in BCB+ oocytes. On the other hand, BCB- oocytes revealed higher mitochondrial activity and an increase in mRNA expression of genes involved in ATP synthesis (ATP5A1), mitochondrial electron transport (FL405), calcium ion binding (S100A10) and growth factor activity (BMP15). Mohammadi-Sangcheshmeh, A. (2011b) using equine oocytes, showed a greater proportion of expanded COC (71% vs. 50%), maturation rate (59% vs. 29%), cleavage rate (46% vs. 29%), and blastocyst (9% vs. 1%) percentage in BCB+ compared with BCB- oocytes. Regarding the gene expression, eight genes were significantly higher for BCB+ oocytes (ATPV6E, IF-3, TFAM, DNMT1, STAT3, Aurora-A, ODC1, and CKS2) whereas BCB- oocytes showed higher expression of COX1. In porcine, Egerszegi I. *et al.* (2010), observed that BCB+ oocytes were characterized by a high mitochondrial activity with a homogeneous distribution while BCB- oocytes had a low mitochondrial activity with a heterogeneous distribution. In addition, BCB+ oocytes showed an increase in maturation (82% vs. 7%) and fertilization (17% vs. 0%) respect BCB- porcine oocytes.

2.3. *Parameters affecting oocyte quality*

2.3.1. Age of the donor.

The age of the female donor is an important issue in ART evidencing a reduced developmental competence when prepubertal donors are used. Armstrong *et al*.(1997) suggested that the optimal age to collect oocytes from prepubertal lambs is between 4 and 6 weeks of age as this is the time of most follicular responsiveness. In a sheep study, in which oocytes were *in vivo* fertilized and flushed from oviducts of prepubertal or adult ewes and transferred to adult recipients, showed that only 33% of the zygotes transferred from prepubertal donors resulted in birth compared to 73% from adult oocyte donors (Quirke and Hanrahan. 1977). Ledda *et al*.(2001) reported that although prepubertal sheep oocytes reach the MII stage at the same percentages as adult animals, they show a lower level of MPF compared to adult ones. In addition, lambs produce 16% of cleaved zygotes that reaches the blastocyst stage, significantly lower than the 34% produced by adult sheep donors (O'Brien et al. 1997).

Comparable results were found in other species. In cow, Revel *et al.* (1995) reported similar rates of IVM, IVF and cleavage for calf and cow oocytes, but after 7 days of IVC, the blastocyst percentage was significantly lower for calf than for cow oocytes (10% vs. 20%, respectively). In pig, Grupen *et al*. (2003) showed that the rates of cleavage (92% vs. 73%) and blastocyst formation (57%vs. 38%) were higher for adult oocytes than for prepubertal oocytes and that the blastocysts derived from adult oocytes had more trophectoderm cells (43 vs. 30) and total cells (51 vs. 36) than those derived from prepubertal oocytes.

This drastic reduction in blastocyst development of prepubertal donors is generally attributed to an incomplete cytoplasmic maturation, traduced in the failure of the sperm to penetrate and decondensate, inability to form normal male pronuclei, failure to block polyspermy, early cleavage failure and failure to reach or survive the transition from maternal to embryonic genome expression among others [reviewed by (Armstrong. 2001)].

2.3.2. Follicular size.

Several authors concluded that there is a correlation between the follicle diameter and the oocyte size and its competence (Martino et al. 1994; Fair et al. 1995; Ledda et al. 1999). In prepubertal goat and ovine the most competent oocytes are the ones coming from follicles bigger than 3mm (Martino et al. 1994; Cognie et al. 1998). In adult goats, Crozet *et al.* (2000) obtained a higher percentage of blastocysts (6% ,12% and 26%) using oocytes from small (2-3 mm), medium (3.1-5 mm), large (> 5 mm) follicles, respectively. Comparing adult and

prepubertal pig oocytes, Bagg *et al.* (2007) showed that rates of blastocyst development after parthenogenetic activation of adult oocytes from three different follicles sizes (3mm, 4mm, and 5-8 mm) were similar (approximately 55%), whereas rates from prepubertal oocytes increased with the increasing follicle size (17%, 36% and 55%, respectively). They concluded that the low developmental competence in prepubertal porcine oocytes is associated with a greater proportion of 3 mm follicles and not to an effect of the female age. In our laboratory we have previously described in prepubertal goat that most of the follicles present in the ovaries were between 2.5 and 3 mm and only 1.1% of follicles per ovary were larger than 3 mm (Martino et al. 1994). More recently, we have reported a higher oocyte size (128 μm vs. 125 μm), higher percentages of TUNEL positive (43% vs. 24%), higher cleavage (48% vs. 23%) and higher blastocyst rates (20% vs. 4%) in oocytes deriving from follicles with diameter >3 mm than from oocytes deriving from follicles with diameter <3mm. Blastocyst mean cell number did not show significant differences between the different follicular groups (123 vs. 104 blastomeres) (Romaguera et al. 2010). As well, significant differences were found when comparing blastocyst rates of oocytes recovered from follicles with diameter <3 mm of prepubertal goats to those from adult goats (5% vs. 21%, respectively). However, when prepubertal goat follicles of >3mm were used, no differences were found comparing to adult oocytes (18%) (Romaguera et al. 2011). In addition, Kauffold *et al.* (2005), showed an increase in blastocyst production in oocytes coming from calf follicles with diameter > 8mm (47%) than from follicles of < 8 mm (<15%). In addition, they found no differences in blastocyst production when comparing oocytes from calf (47%) and cow (59%) from a follicle diameter bigger than 8mm.

2.3.3. Oocyte size.

It has been shown that oocyte size is closely related to the developmental competency. Gandolfi *et al.* (1998) showed differences in oocyte size between cow (123 μm) and calf (118 μm) oocytes. This difference in oocyte diameter was reflected in a significant decrease in protein synthesis after 9 h of IVM in calf oocytes, while in cow adult it was detected after 24 h. In prepubertal goat, oocytes from different diameter (< 110, 110-125, 125-135 and > 135 μm) showed a positive correlation to the percentage of oocytes reaching MII stage after IVM (0%, 21%, 58% and 78%, respectively) and the percentage of blastocysts obtained at 8 days post-insemination (0%, 0%, 2% and 13%, respectively). Also, the protein expression of p34[cdc2] and the MPF activity increased in each oocyte category after 27 h of maturation (Anguita et al. 2007).

Differences in classification of oocyte size were reported in cattle that could be attributed to differences in cattle breeds and methods of measuring oocyte diameter. Hyttel *et al.*(1997) showed that even thought oocytes of 100 μm had full competence for the resumption of meiosis,

they produce significant lower percentages of blastocysts (30%) in comparison to oocytes with a size larger than 110 μm (60%). Otoi et al. (1997) classifying oocytes in six categories (<110, 110-114, 115-119, 120-124, 125-129 and ≥ 130 μm) concluded that bovine oocytes with a diameter larger than 115 μm can reach the meiotic competence, but to acquire fully embryo development competence and reach the blastocyst stage the best diameter of oocytes is from 120 μm (6%, 9%, 16%, 24%, 30%, 0% blastocyst, respectively). Arlotto *et al.* (1996) classified oocytes in 4 categories (95-104, 105-114, 115-124, 125-134 μm) with a diameter average of 122.5 μm, concluded that bigger oocytes produce more blastocyst (10%, 23%, 34%, 39%, respectively). Similar results were found in buffalo in which the mean diameter of oocytes was 146.4 μm (<126, 127-144, 145-162, >163 μm) and the rate of blastocyst production *in vitro* was significantly higher in oocytes with diameters greater than 145 μm [0%, 1%, 7.3%, 10.4%, respectively; (Raghu et al. 2002a)].

2.4. Improving oocyte quality using *in vitro* media.

As was previously stated, oocytes acquire developmental competence sequentially during follicle growth, reaching the fully meiotic competence at the early antral stage of the follicle growth when they have accumulated all the regulating molecules in sufficient amounts to enable resumption of meiosis. So, reaching the oocyte competency is closely correlated to the oocyte size which is associated with follicle diameter. Since follicles of juvenile animals are usually smaller than those of adults, it is difficult to separate maternal age effects from those related to follicle diameter [revised by (Armstrong. 2001)].

Juvenile donors produce a high amount of small diameter follicles with incompetent oocytes. Consequently, Wu *et al.* (2006) using a growth medium during the IVM of porcine oocytes from small diameter follicles, showed an increase in oocyte nuclear maturation (55% vs. 36%) and developmental competency (13% vs. 3%) of these oocytes compared to those matured in the conventional direct oocyte maturation system. This media consist in a more growth-supporting and less maturation-promoting environment during the first phase of the oocyte maturation with the addition of Insuline Transferrine Selenium and Ascorbic Acid.

2.4.1. Insuline Transferrine Selenium (ITS).

Insulin is a polypeptide hormone that promotes the uptake of glucose and amino acids and may have mitogenic effects. It has also been reported that insulin stimulates the proliferation and steroidogenesis of granulosa and theca cells (Campbell et al. 1995; Spicer and Echternkamp. 1995; Duleba et al. 2001). In the ovarian tissue, insulin stimulates granulosa cell progesterone secretion and granulosa cell luteinization (Channing et al. 1976). Insulin and Insulin Growth

Factor (IGF) produce an increase in the developmental potential of porcine oocytes and embryos during IVM and IVC (Tsafriri and Channing. 1975). In the mouse, an increase of the protein synthesis was detected in the presence of insulin at the compacted morulae stage of development (Rao et al. 1990) when the insulin receptor appears (Harvey and Kaye. 1988).

Transferrin and selenium are essential trace elements and may have antioxidant activity in biological systems (Wu et al. 1973; Gutteridge. 1986). Transferrin is a glycoprotein that binds iron very tightly but reversibly. It has a molecular weight of around 80 kDa and contains 2 specific high-affinity Fe^{3+} binding sites. The affinity of transferrin for Fe^{3+} is extremely high but decreases progressively with decreasing pH below neutrality (Crichton and Charloteaux-Wauters. 1987). Selenium can be found in the body as part of at least 25 selenoproteins (Kryukov et al. 2003). Those selenoproteins are considered to be involved in the regulation of various physiological functions including antioxidant protection, redox regulation of gene expression, thyroid metabolism, and sperm structure integrity maintenance (Surai. 2002).Insulin–transferrin–selenium together could be supplemented in both complex and non-complex media. In pig, the addition of ITS to the *in vitro* maturation medium promote nuclear maturation [79% vs. 54%; (Hu et al. 2011)], decreased polyspermy (35% vs. 57%) and increased male pronuclear formation (73% vs. 52%) compared to the non addition (Jeong et al. 2008). In buffalo, the ITS increased blastocyst number (Raghu et al. 2002b). Cordova *et al.*(2010) showed that supplementing the calf maturation medium with ITS plus L-ascorbic acid during the first 12 h of IVM improves cytoplasmic maturation and the developmental competence respect oocytes matured without ITS plus L-ascorbic acid (20% vs.12%, respectively).

2.4.2. Ascorbic acid.

Vitamins are important nutrients involved in multiple cell functions, including mammalian reproduction, not only as cellular antioxidants, but also as modulators of intracellular and extracellular biochemical processes [revised by (Tao et al. 2004)]. The oxidative stress is detrimental to granulosa cells and oocytes, it is for this reason that the use of chemically defined media containing vitamins such as L-ascorbic acid (vitamin C) and α-tocopherol (vitamin E) could improve oocyte quality (Eppig et al. 2000; Tao et al. 2004). L-ascorbic acid is necessary for collagen synthesis, promotes steroidogenesis and acts as an antioxidant in many biological processes [revised by (Murray et al. 2001)]. In addition, Murray *et al* (2001) showed that even thought L-ascorbic acid had no effect on follicles growth or on estradiol production, it significantly reduced apoptosis. In sheep oocytes, Natarajan *et al.* (2010) showed that the addition of 50 μM L-ascorbic acid to the embryo culture medium significantly increased the rates of morulae (41%), blastocysts (20%) and blastocyst total cell number (108 cell) when

compared to control (30%, 13%, 92, respectively). However, no effect was found when supplementing the *in vitro* maturation medium with different concentrations of L-ascorbic acid.

Chapter 3:

Objectives

1- To develop the methodology of the Brilliant Cresyl Blue stain as a noninvasive technique to select more competent oocytes for in vitro blastocyst production in sheep.

2- To study sheep oocyte quality by assessing mitochondria distribution and activity, genes expression, ATP and MPF activity in selected BCB oocytes.

3- To increase the *in vitro* blastocyst production of the prepubertal sheep oocytes by improving the oocyte competence using a growth media during the IVM.

4- To study the response in blastocyst production of BCB selected oocytes after IVF (in vitro fertilization), ICSI (Intracytoplasmic Sperm Injection) and PA (Parthenogenetic Activation).

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

REPRODUCTION

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 μM) and classified according to their cytoplasm colouration as grown BCB+ (blue cytoplasm) and growing BCB− (colourless cytoplasm). Staining oocytes with 13 μM BCB during 60 min allows selection of (BCB+) the largest (123.66 μm) 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 μm, 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 μm 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 road (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 ($\pm$ 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 $\pm$ 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.

	N	Cell number (mean±s.e.m.)			Percentage/total cells (*n*)		
		Total	TE	ICM	ICM	TE	ICM:TE ratio
BCB+	24	69.71 ± 6.19[a]	50.88 ± 5.06[a]	18.83 ± 1.77[a]	27.01	72.99	1:2.70
BCB−	22	45.91 ± 3.35[b]	33.36 ± 3.16[b]	12.55 ± 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; $P<0.05$).
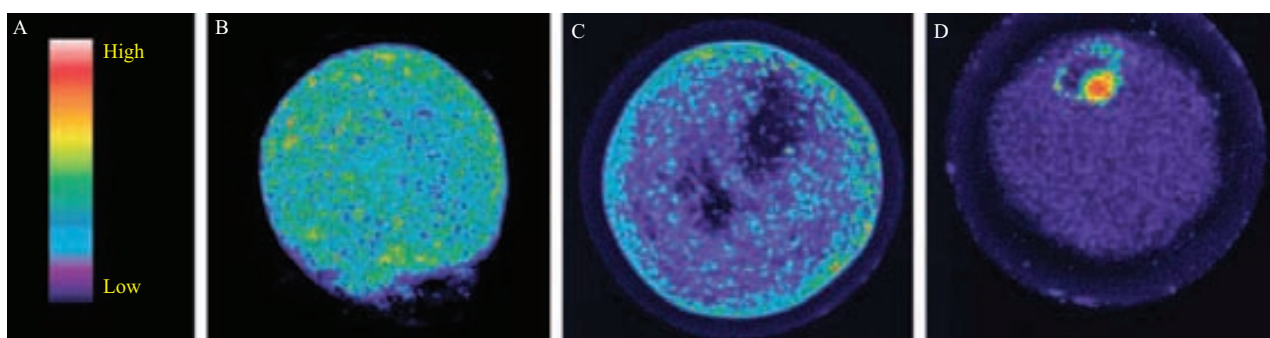
Figure 1 Representative images of active mitochondrial distribution of prepubertal sheep oocytes taken by a confocal microscope. Images were spectrally (A) coded to represent staining intensity (red is the highest intensity). Representative images of (B) homogeneous, (C) peripheral and (D) polarised 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)
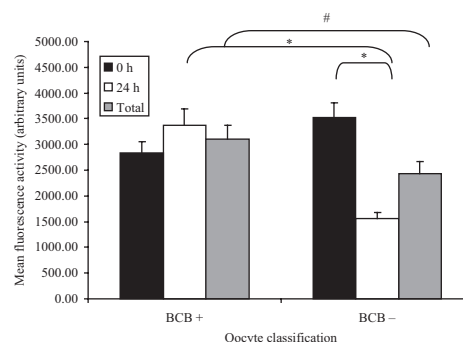

Figure 2 Mitochondrial activity of BCB selected oocytes assessed by fluorescence intensity before (0 h) and after IVM (24 h). Different symbols (#, *) indicate significant differences (*$P<0.0001$; #$P<0.05$).
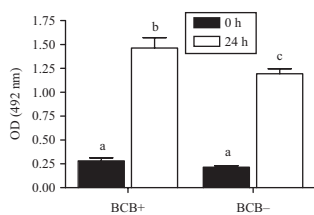
Figure 3 MPF activity in BCB-exposed prepubertal sheep oocytes before (0 h) and after IVM (24 h). MPF activity is expressed as OD at 492 nm. [a,b,c]Indicates a significant difference in kinase activity among oocyte groups.

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
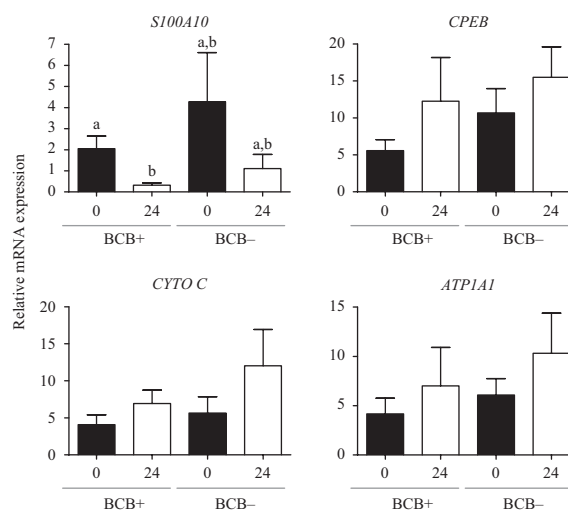

Figure 4 Relative mRNA expression of *S100A10, CPEB, COX1* and *ATP1A1* transcripts before (0 h) and after IVM (24 h) of prepubertal sheep BCB selected oocytes. Different letters (a, b) indicate significant differences ($P < 0.05$: one-way ANOVA with Tukey's multiple comparison post-test to compare the 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 μM) 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% CO_2 in 500 μl of maturation medium covered with mineral oil. Maturation medium consisted of TCM-199 supplemented with 5 μg/ml LH, 5 μg/ml FSH, 1 μg/ml 17β oestradiol, 10 ng/ml epidermal growth factor, 0.2 mM sodium pyruvate, 2 mM glutamine, 100 μM 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 μl 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% CO_2 and 5% O_2 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 μl 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 μg/ml propidium iodide). Immediately afterwards they were transferred to solution 2 (100% ethanol with 25 μg/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 μM 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, maturated 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 μM 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 μg/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 (Espectral 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 (SLYSSPGGAYC). 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	TCATTCTTCGCCAAAAGCACTC		140
	Antisense	AGCCCATATCCTTGTCGTATCC		
18S	Sense	AGAAACGGCTACCACATCCAA	DQ222453.1	90
	Antisense	CCTGTATTGTTATTTTTCGT		

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

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Effect of insulin transferrin and selenium and Ascorbic Acid in maturation media on embryo development, MPF activity and ATP content of prepubertal sheep oocytes selected by brilliant cresyl blue test.

1 **Effect of insulin transferrin selenium (ITS) and Ascorbic Acid in maturation**

2 **media on embryo development, MPF activity and ATP content of prepubertal**

3 **sheep oocytes selected by brilliant cresyl blue (BCB) test.**

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11 **Running Head:** Growth medium on BCB prepubertal sheep oocytes

12

13   **Abstract**

14   Oocytes from prepubertal females are small and with reduced embryo development. In

15   pig, Wu *et al.* (Biol. Reprod. 2006; 75(4): 547-554) increased blastocyst production of

16   small oocytes using a growth medium (GM) during the first 24 hours of IVM. GM was

17   composed by low hormone concentration and the addition of Insulin Transferrin

18   Selenium (ITS) and ascorbic acid (AA). The aim of this study was to test in prepubertal

19   sheep oocytes the GM and moreover to test a modified medium (MM) composed by the

20   addition of ITS and AA to a conventional IVM. Immature oocytes were exposed to

21   Brilliant Cresyl Blue (BCB) and classified according to their cytoplasm coloration as

22   grown BCB+ (blue cytoplasm) and growing BCB- (colorless cytoplasm). The BCB+

23   and control oocytes (oocytes not exposed to BCB) were matured in a conventional

24   medium (CM) for 24 hours. BCB- oocytes were placed in different IVM groups: 1) GM

25   for 12 h and then 12 h in CM. 2) GM for 12 h and 12 h in MM, 3) GM for 24 h and 4)

26   MM for 24 h. There were no differences in blastocyst production among BCB- groups

27   and the control group (3.7, 4.8, 1.3, 3.1% and 4.3%, respectively) but, BCB+ oocytes

28   development was higher (9.1%). MPF activity and ATP content were assessed before

29   and after IVM. MPF and ATP increased in all groups ($P<0.001$) after *in vitro*

30   maturation. In conclusion, the lack of response in BCB- oocytes could be due to their

31   early stage of growth.

32

33 **Introduction**

34 Ovaries from prepubertal animals have high percentage of antral follicles with a

35 diameter smaller than 3 mm (Martino et al. 1994). There is a direct and positive

36 relationship between follicle size, oocyte diameter and oocyte competence to develop up

37 to blastocyst stage (Gilchrist et al. 1995; Barnes and Sirard. 2000). In prepubertal goats,

38 oocytes with a diameter bigger than 125 μm produced higher percentage of blastocyst

39 than the smallest ones after IVF (Anguita et al. 2007) and ICSI (Jimenez-Macedo et al.

40 2007). Using a non invasive technique like Brilliant Cresyl Blue (BCB) test can be a

41 suitable method for selection of larger and more competent oocytes (Roca et al. 1998;

42 Rodriguez-Gonzalez et al. 2002; Alm et al. 2005). The BCB test determines the

43 intracellular activity of glucose-6-phosphate dehydrogenize (G6PDH), a pentose

44 phosphate pathway enzyme. It is particularly active in developing oocytes with a clear

45 decrease in activity when the oocytes have finished their growth phase. Fully grown

46 oocytes show a decreased G6PDH activity, remaining with blue colored cytoplasm

47 following the uptake of BCB (BCB+), while growing oocytes show a colorless

48 cytoplasm (BCB-). We have previously showed the effectiveness of BCB stain in

49 prepubertal sheep oocytes by selecting larger oocytes (123.66 μm) which produced

50 higher blastocyst rates (24%) in BCB+ than in BCB- group (106.82 μm and 9%,

51 respectively) (Catalá et al. 2011). The inconvenient is that only 10 to 20 % of

52 prepubertal sheep (Catalá et al. 2011) and goat (Rodriguez-Gonzalez et al. 2002)

53 oocytes are BCB+, while in adult females the percentage of BCB+ oocytes is higher

54 [goat: 85%, (Kątska-Książkiewicz et al. 2007) buffalo: 60% (Manjunatha et al. 2007);

55 cattle: 60% (Alm et al. 2005)]. In pig, Wu *et.al* (2006) demonstrated that placing the

56 small follicle-derived COCs in a more growth-supporting and less maturation-

57 promoting environment in the first phase (24 h) of *in vitro* maturation culture allows

54

58 higher blastocyst development compared with the conventional maturation-stimulating

59 system. Wu's hypothesis was that oocytes that were still at their last stage of growth and

60 have been liberated from the follicle could not complete meiosis since their intracellular

61 environment is not completely ready for the process. So, adding Ascorbic Acid (AA)

62 and Insulin Transferrin Selenium (ITS) as supplements and reducing FSH and LH

63 (1/250 of concentration used in the IVM medium) during the first 24 h (called Growth

64 Medium, GM) and subsequently transfer them into conventional IVM for further 20 h

65 improved significantly the embryo development of the smallest oocytes.

66 Oocyte maturation involves the activation of various signal transduction pathways that

67 converge to activate maturation promoting factor (MPF). In prepubertal goat oocytes we

68 have shown a positive relationship between oocyte diameter, blastocyst development

69 and MPF activity (Anguita et al. 2007). In prepubertal sheep oocytes, MPF and

70 mitochondria activity were higher in BCB+ oocytes than BCB- oocytes assessed after

71 24 h of IVM (Catalá et al. 2011). Related to mitochondria activity significant increase

72 of ATP content in oocytes during maturation has been reported in pig (Brevini et al.

73 2005) and cattle (Stojkovic et al. 2001), and this ATP increase is correlated with the

74 success rates in embryo development in cattle (Stojkovic et al. 2001).

75 The aim of this study is to improve embryo development of small and growing (BCB-)

76 oocytes of prepubertal sheep. Thus, in Experiment 1, according to the results of Wu *et*

77 *al.* (2006), we will test the supplementation with ITS and Ascorbic Acid (AA) in a

78 medium with low concentration of hormones (called Growth Medium, GM) during the

79 first 12 h of the IVM. In another study we will test ITS and AA supplementation in the

80 conventional IVM during the 24 h of culture. In Experiment 2, we will analyze the MPF

81   activity and the ATP content in control, BCB+ and BCB- before (0 h) and after 24 h of

82   IVM.

83

84   **Materials and methods**

85   *Chemical*

86   All chemicals were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA)

87   unless otherwise is specified.

88   *Oocyte collection*

89   Ovaries from prepubertal ewes (3-6 month old), were obtained from a local abattoir and

90   transported to laboratory in sterile Dulbecco's phosphate-buffered saline (PBS) held at

91   34-37 C and were washed in PBS containing antibiotic-antimicotic (AB, Gibco cat Nro

92   14240-062). Cumulus oocytes complexes (COCs) were recovered by slicing the surface

93   of the ovary in HEPES-buffered TCM199 medium with 0.5 g/l Fraction V Bovine

94   Serum Albumine (BSA). Oocytes with two or more complete layers of compact

95   cumulus cells and homogeny cytoplasm were used.

96   *Brilliant Cresyl Blue test*

97   Immediately after collection, oocytes were washed three times in mPBS (PBS

98   supplemented with 1 g/l glucose, 36 mg/l sodium piruvate, 0.5 g/l BSA, 0.05 g/l

99   Gentamicine,) and exposed to 13 μM of BCB diluted in mPBS during 1 h at 38.5 C in a

100   humidified air atmosphere. After BCB incubation, oocytes were washed three times in

101   mPBS and classified into two groups depending on their cytoplasm coloration: oocytes

102   with a blue cytoplasm or hypothetically grown oocytes (BCB+) and oocytes without a

103   blue cytoplasm or hypothetically growing oocytes (BCB-).

104   *In vitro maturation of oocytes*

105   Groups of 35-40 COCs of BCB+, BCB- and control (oocytes exposed during 1 h to

106   mPBS without BCB colorant), were washed and culture in 500 µl of the IVM medium

107   under mineral oil according to experimental treatments and culture at 38.5 C in a

108   humidified air atmosphere with 5% CO_2. Three different maturation mediums were

109   analyzed.

110   **CM**: Conventional maturation medium consisting in TCM199 supplemented with 5

111   µl/ml FSH, 5 µg/ml LH, 1 µl/ml 17β estradiol.

112   **GM:** Growth maturation medium, consisting in TCM 199, 0.04 µl/ml FSH, 0.04 µl/ml

113   LH, 0.004 µl/ml estradiol, 5 µl/ml of ITS solution (Invitrogen, Cat No: 41400-045) and

114   100 µg/ml AA (adapted to sheep protocol, from Wu *et al* (2006).

115   **MM** Modified Maturation medium, consisting in CM medium supplemented with 5

116   µl/ml ITS and 100 µg/ml AA.

117   All media were also supplemented with 10 ng/ml EGF, 0.2 mM sodium pyruvate, 2 mM

118   ʟ-glutamine, 100 µM Cysteamine, 10% (v/v) FBS (Fetal Bovine Serum), 2% (v/v)

119   antibiotic-antimitotic.

120   *In vitro Cdc2 kinase activity assay*

121   Before and after IVM, groups of 20 oocytes BCB+, BCB- and control group matured

122   during 24 h in CM and 20 oocytes BCB- oocytes matured during 24 h in GM (three

123   replicates) were washed three times in PBS, and placed in tubes containing 5 μl of lysis

124   buffer (50 mM Tris–HCl pH 7.5, 0.5 M NaCl, 5 mM EDTA, 0.01% Brij35, 1 mM

125   PMSF, 0.05 mg/ml leupeptin, 50 mM 2-mercaptoethanol, 25 mM β-glycerophosphate, 1

126   mM Na-orthovanadate). The samples were frozen in liquid nitrogen and sonicated three

127   times at 1 C for 25 sec. Cell extracts were stored at −80 C until use.

128   Cdc2 assay was performed using the MESACUP cdc2 kinase assay kit (MBL, Japan)

129   following the manufacturer's protocol. Five micro-liters of oocyte extract were mixed

130   with 10X cdc2 Reaction Buffer (25 mM Hepes buffer pH 7.5, 10 mM $MgCl_2$) and 10%

131   biotynilated MV Peptide (SLYSSPGGAYC). The phosphorylation reaction was started

132   adding 0.1 mM ATP (Sigma, USA), in a final volume of 50 μl. The mixture was

133   incubated at 30 C for 30 min. The reaction was finished by adding 200 μl of

134   phosphorylation Stop Reagent (PBS containing 50 mM EGTA). The phosphorylated

135   MV peptide was detected by ELISA at 492 nm.

136   *Adenosine 5'-thriphosphate (ATP) analysis.*

137   The ATP analysis was performed using the Adenosine 5'-thriphosphate bioluminescent

138   somatic cell assay kit (FLASC), as manufacture's protocol. Briefly, groups of 6 BCB+

139   BCB- and control oocytes before and after IVM were completely denuded by gently

140   pipetting and placed into an eppendorf with 200 μl ultrapure water and stored at -80 C

141   until use.

142   For reaction analysis, 0.1 ml of ATP assay mix working solution was added to a

143   reaction vial during 3 min to hydrolyze any endogenous ATP. Then 0.05 ml of ultrapure

144   water and 0.05 ml of sample were added to a separate vial containing 0.1 ml of 1x

145 somatic cell releasing reagent. Finally 0.1 ml of this solution was transferred to the

146 reaction vial and immediately measured the light emitted with a luminometer.

147 *In vitro fertilization and embryo culture*

148 Fresh semen, obtained of 3 rams of proven fertility, was keep at room temperature (25

149 C) during 90 min. Highly motile spermatozoa were selected by using Ovipure density

150 gradient (Nidacon EVB S.L.) and fertilized with $1x10^6$ spermatozoa/ml. Matured

151 oocytes were partially denuded and transferred into fertilization medium consisting of

152 Synthetic Oviductal Fluid (SOF) supplemented with 10% of estrous sheep serum.

153 Fertilization was carried out in drops of 50 μl of SOF medium with a maximum of 15

154 oocytes per drop and incubated during 20 h at 38.5 C, 5% CO_2 and 5% O_2 in humidified

155 atmosphere.

156 After IVF, groups of 6 presumptive zygotes were completely denuded by gently

157 pippeting and cultured for 8 days in 20 μL culture drops consisting of SOF medium

158 supplemented with 10% (v:v) FBS at same atmosphere conditions.

159 *Experiment 1*

160 In experiment 1 we analyzed the effect of using a 2 step IVM protocol in BCB- oocytes

161 in 7 replicates. GM media was used in BCB- oocytes during the first 12 h of IVM. After

162 that, oocytes were placed for another 12 h in CM and MM to complete the 2 step

163 protocol as Wu *et al* (2006) described it. This experiment was performed with 5

164 treatments: 1) Control (not BCB treated oocytes) matured for 24 h in CM 2) BCB+

165 oocytes matured for 24 h in CM and 3) BCB- oocytes matured for 24 h in CM. 4) BCB-

166 matured during 12 h in GM and 12 h in CM and 5) BCB- oocytes matured during 12 h

167 in GM and 12 h in MM. After IVM, all of experimental groups were fertilized and

168 cultured for 8 days after insemination.

169 In this experiment moreover we analyzed the effect of ITS and AA during the whole

170 process of *in vitro* maturation (1 step maturation protocol). This experiment was

171 performed with 3 treatments: 1) Control oocytes (not BCB treated) matured for 24 h in

172 CM 2) BCB+ oocytes matured for 24 h in CM and 3) BCB- oocytes matured for 24 h in

173 CM 4) BCB- oocytes matured during 24 h with GM and 5) BCB- oocytes matured

174 during 24 h with MM.

175 *Experiment 2*

176 We studied the effect of the conventional 1 step maturation protocol using CM as

177 maturation medium in Control, BCB+ and BCB- group by analyzing the activity of the

178 MPF (3 replicates of 20 oocytes each) and ATP (3 replicates of 6 oocyte each) content.

179 MPF and ATP were also analyzed in oocytes matured in GM and MM.

180 *Statistical analysis*

181 Analysis among treatments (IVC) were performed by Fisher's exact test and for MPF

182 activity and ATP analysis One-way ANOVA in GraphPad Prism v 3 (GraphPad

183 Software, San Diego California USA) was used.

184

Results

185 **Results**

186 *Experiment 1.*

187 The results of embryo development using different IVM media are shown in Table 1.

188 Using the conventional medium (CM), the percentage of blastocysts obtained was

189 significantly higher in BCB+ than BCB- and control oocytes (P<0.01). Comparing

190 BCB- oocytes matured in different media in 1 and 2- steps protocol, cleavage rate was

191 improved in GM and MM matured oocytes compared to conventional matured BCB-

192 oocytes however, we did not find differences in blastocyst production among groups.

193 Regardless of IVM treatment, BCB- oocyte groups developed to blastocyst stage a

194 lower percentage than BCB+ oocytes.

195 *Table 1. In vitro embryo development of prepubertal sheep oocytes selected by Brilliant*

196 *Cresyl Blue (BCB) and matured in different IVM media (7replicates).*

Treatments	Inseminated oocytes	48 post insemination	Embryo Development at 8 days post insemination	
		Cleavage,n(%)	Blastocyst /total,n(%)	Blastocyst /Cleavage(%)
CM. Control	282	192 (68.1)[a]	12 (4.3)[a]	6.3[a]
CM. BCB+	243	166 (68.3)[a]	22 (9.1)[b]	13.3[b]
CM. BCB-	216	125 (57.9)[b]	5 (2.3)[a]	4.0[a]
2 Steps IVM of BCB- oocytes				
12 h GM+12 h CM	268	167 (62.3)[a]	10 (3.7)[a]	5.9[a]
12 h GM+ 12 h MM	208	138 (66.4)[a]	10 (4.8)[a,b]	7.3[a,b]
1 Step IVM of BCB- oocytes				
GM	238	103 (43.3)[c]	3 (1.3)[a]	2.9[a]
MM	227	180 (79.3)[d]	7 (3.1)[a]	3.9[a]

197 CM: conventional media. GM: growth media. MM: modified media. Values in the same column with

198 different letters [(a, b)] differ significantly (Fisher Test, P<0,05).

199

200

61

201   *Experiment 2.*

202   Results of MPF activity in different prepubertal sheep oocyte groups are represented in

203   figure.1. We could see that there were no differences at collection time (0 h) among the

204   different groups. After IVM, MPF activity showed a significant increase in each group

205   (P<0.001) respect the immature stage. Also, MPF showed a significantly higher activity

206   in BCB+ than in control and BCB- groups (P<0.05).

207
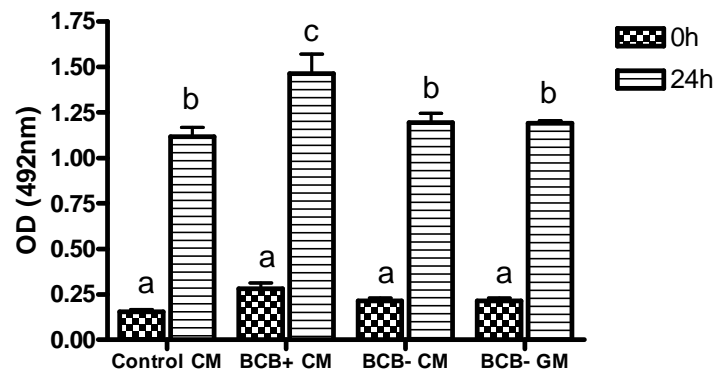
208

209   **Figure 1.** MPF activity in Control and BCB+ oocytes matured in Conventional Medium (Control CM and
210   BCB+ CM) and BCB- oocytes matured in CM and in Growth Medium (BCB-CM and BCB-GM) during
211   24 h (3 replicates) . Analysis was performed before (0h) and after IVM (24h). MPF activity is expressed
212   as OD at 492nm. One-way ANOVA in GraphPad Prism v 3 (GraphPad Software, San Diego California
213   USA) was used. [a,b,c] indicate a significant difference (P<0.05) in kinase activity among oocyte groups.

214   Results of ATP content in different sheep oocytes groups are shown in figure 2. At

215   collection time (0h), control oocytes presented significantly higher ATP content than

216   BCB- group. No differences were found among control, BCB+ and BCB- groups at 0h.

217   In all groups, a significant increase (P<0.001) of the ATP content was observed after

218   IVM. Not differences in ATP content was found among oocyte groups at 24h, in spite

219   of BCB+ oocytes presented an increase in MPF activity (figure 1.) compared to BCB-

220   oocytes at this time. Maturing BCB- oocytes in 1 or 2-steps did not affect the ATP

221   content.
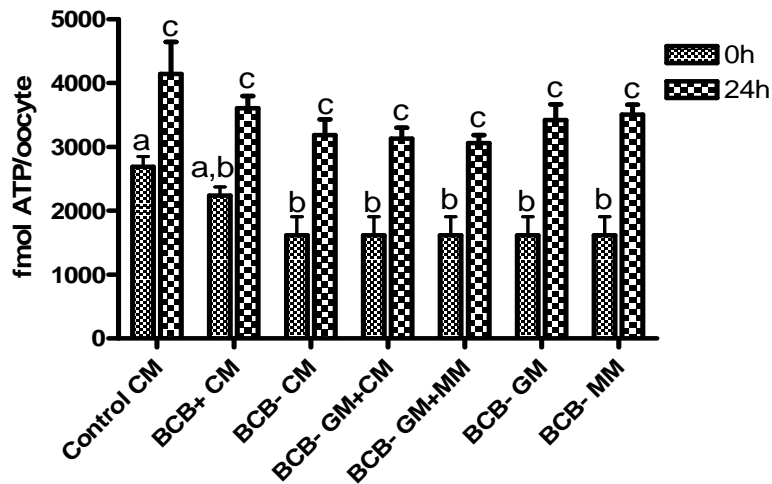
222

223   **Figure 2**: ATP content in prepubertal sheep oocytes before (0h) and after IVM (24h) in different media (3
224   replicates). Control, BCB+ and BCB- oocyte groups were matured in a conventional medium (CM)
225   (Control CM, BCB+ CM and BCB- CM). The rest of BCB- oocytes were matured in 2- steps maturation
226   system composed by Growth medium (GM) and Modified medium (MM) (BCB- GM+CM and BCB-
227   GM+MM) or matured in 1 step of IVM (BCB-GM and BCB- MM). GM One-way ANOVA in GraphPad
228   Prism v 3 (GraphPad Software, San Diego California USA) was used. [a,b,c] indicate a significant
229   difference ($P < 0.05$) in kinase activity among oocyte groups.

230

231   **Discussion**

232   In goat, we have previously shown that oocytes from prepubertal females coming from

233   large follicles (> 3mm diameter) developed up to blastocyst stage at the same

234   percentage than oocytes from adult females (Romaguera et al. 2011). In pigs, Bagg *et al*

235   (2007) obtained the highest blastocyst rate after parthenogenetic activation in oocytes

236   from follicles larger than 5 mm regardless of the female age. They concluded that the

237   low developmental competence in prepubertal porcine oocytes is associated with a

238   greater proportion of small follicles compared to adult ovaries. In agreement, Kauffold

239   *et al* (2005) in cattle did not find differences in blastocysts production between calf and

240   cow if oocytes coming from follicles larger than 8 mm. In conclusion, we suggest that

241   the low embryo development of prepubertal female oocytes is due to high percentage of

242   unfinished growth follicle and oocyte of these ovaries. In order to increase the number

243   of *in vitro* blastocysts obtained from prepubertal ovaries we have tested in lamb oocytes

244   the oocyte Growth Maturation protocol described by Wu *et al* (2006) and different

245   variations of it. Previously, we had shown the utility of the BCB test as a technique to

246   discriminate larger and more competent oocytes (BCB+) from smaller and growing

247   lamb oocytes (Catalá et al. 2011). In the present study we confirmed the significantly

248   higher blastocyst yield of BCB+ oocytes (9.1%) compared to BCB- oocytes (2.3%) and

249   control group (4.3%). Different authors in cattle (Pujol et al. 2004)), pig (Roca et al.

250   1998) and goat (Rodriguez-Gonzalez et al. 2003) have confirmed this usefulness of

251   BCB test to select the more competent oocytes.

252   In the present study we have tested in lamb BCB- oocytes the 2 steps Growth

253   Maturation protocol described by Wu *et al* (2006). We have not found significant

254   differences in blastocyst production between BCB- oocytes matured in the conventional

255   maturation medium (2.3%) and the 2 steps oocyte growth maturation medium (3.7%).

256   In calf oocytes, Cordova *et al* (2010) adding for 12 h Ascorbic Acid (AA) and ITS to

257   the IVM (without changing hormone concentration) found a significantly higher

258   blastocyst production and cytoplasmic maturation assessed by Cicline B protein,

259   cortical granule distribution and chromosome organization than control group. This lack

260   of effect in lamb oocytes in our study could be due to the highly immature BCB-

261   oocytes or to the low hormone concentrations used in GM. Thus, to enhance the

262   beneficial effect of the AA plus ITS supplementation on BCB- lamb oocytes we

263   increased the hormone concentrations to a conventional level. This new medium was

264   called Modified Medium (MM). Here again, we did not find statistically significant

265   differences in blastocyst rate (4.8%) but we have observed an increase compared to

266   conventional medium (2.3%). ITS and AA supplementation for 24 hours, with

267   conventional or low hormone concentrations, did not improved blastocyst rate (3.1 and

268   1.3%, respectively) and this was significantly lower than BCB+ oocytes (9.1%). Several

269   authors have shown the positive effect of ITS and AA on embryo development. In pig,

270   Jeong *et al.*(2008) found a significantly increase in blastocyst production by ITS

271   addition during *in vitro* maturation in a chemically defined and porcine Folliclular-Fluid

272   (pFF) media. Although the best results of blastocyst production were found in pFF

273   medium. Also in buffalo, Raghu *et al*. (2002) concluded that the addition of ITS in a

274   maturation medium supplemented with gonadotrophins, EGF and serum improved

275   blastocyst production. Various physiological roles of ITS in oocyte and embryo

276   development have been postulated. Insulin stimulates the synthesis of DNA, RNA,

277   protein and lipid, and the utilization of glucose, and thereby regulates cellular functions

278   by its action on the plasma membrane, cytoskeleton, intracellular enzymes and the

279   nucleus (Harvey and Kaye. 1988; Rao et al. 1990). Transferrin, a serum globulin, acts as

280   a detoxifying protein by removing toxic metals from the medium (Barnes and Sato.

281   1980). Selenium may help to combat the oxidative stress by regulating the activity of

282   glutathione peroxidase (Stadtman. 1974; Lee et al. 2001). In our study, BCB- oocytes

283   matured in a complex medium with gonadotrophins, estradiol, EGF, serum, ITS and AA

284   not increased its embryo development competence. In these BCB- oocytes, we did not

285   find any effect of hormones concentrations on embryo development. This lack of effect

286   of maturation media on BCB- oocytes is also represented by MPF activity and ATP

287   content. In our study, MPF activity after 24 h of IVM is significantly higher in BCB+

288   oocytes than in control and BCB- oocytes. No differences were found in BCB- oocytes

289   matured in conventional or ITS and AA supplemented media. In our previous study

290   (Catalá et al. 2011) we have observed higher MPF and mitochondrial activity in BCB+

291   than in BCB- oocytes after 24 h of IVM, speculating that there could be a positive

292   relation between the ATP produced by the active mitochondria and the ATP content

293   needed to phosphorylate p34cdc2 and activate the MPF complex. In the present study

294   we observed a significantly increase in ATP content after 24 h of *in vitro* maturation

295   compared to oocyte at collection time (0 hours) in all of oocytes regardless of

296   maturation media and oocyte quality. However, we did not find differences among the

297   different oocyte groups.

298   Higher MPF activity has been observed in oocytes from adult females compared to

299   oocytes from prepubertal females in sheep (Ledda et al. 2001) and cattle (Salamone et

300   al. 2001). Moreover, assessing MPF activity according to oocyte diameter, Anguita *et

301   al.* (2007) showed a positive correlation among MPF activity, oocyte diameter and

302   embryo development competence in prepubertal goat oocytes.

303   Ptak *et al*. (2006) comparing oocytes from prepubertal sheep classified as small and

304   adult-size oocytes concluded that small oocytes lack both cytoplasmic and nuclear

305   competence with a fibrilogranular nucleolus in small oocytes and compact nucleolus in

306   large oocytes. Moreover blastocyst development after ICSI was 3% in small and 15% in

307   large prepubertal sheep oocytes. In adult sheep, Mohammadi-Sangcheshmeh et al (

308   2011) observed that after IVM, BCB+ oocytes reached MII stage at a higher ratio than

309   BCB- oocytes (79 and 52%, respectively) and blastocyst development (34 and 4%,

310   respectively). In our study with lamb oocytes, the reason for the failure of BCB- oocytes

311   to increase blastocyst production after a growth-maturation system and to the ITS and

312   AA supplementation could be due to their reduced meiotic competence and abnormal

313   cytoplasmic maturation.

314 In conclusion, the BCB staining has allowed us to confirm the higher percentage of

315 blastocyst obtained of BCB+ oocytes and their higher MPF activity compared to BCB-

316 oocytes. However, despite changes in the maturation media, the BCB-oocytes have not

317 improved MPF activity, ATP content and embryo development up to blastocyst. These

318 results may be due to the early stage of growth of these oocytes that unable them to

319 respond to maturation supplementation with ITS and AA.

320

321 Conflicts of interest

322 The author does not have any conflict of interest to declare.

323 Author contributions

324 All authors were involved in all phases of the research and paper.

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Effect of oocyte quality on blastocyst development after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in a sheep model.

ORIGINAL ARTICLE: REPRODUCTIVE BIOLOGY

Effect of oocyte quality on blastocyst development after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in a sheep model

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Objective: To compare blastocyst production, after IVF and ICSI, from sheep oocytes of various quality. Sham-injected oocytes and parthenogenetic activated oocyte groups were considered as control.

Design: Prospective experimental study.

Setting: University.

Animal(s): Three- to 6-month-old sheep.

Intervention(s): Oocyte quality was assessed with the use of brilliant cresyl blue (BCB) stain. Adenosine triphosphate content was measured. Intracytoplasmic sperm injection and IVF were performed and blastocyst development and cell numbers were analyzed.

Main Outcome Measure(s): Adenosine triphosphate content, embryo development and blastomere numbers.

Result(s): After IVF, BCB-stained (BCB+) oocytes developed up to the blastocyst stage at higher percentages and with more cells per embryo (24.1% vs 4.0% and 69.7 vs 43.9, respectively) than unstained (BCB−) oocytes. Using intracytoplasmic sperm injection, no differences were found in blastocyst production (14.3% vs 11.8%) and number of cells per embryo (71.1 vs 54.3) between BCB+ and BCB− oocytes. Adenosine triphosphate content was higher before in vitro maturation than after in both types of oocytes. Brilliant cresyl blue–stained oocytes had more adenosine triphosphate content than BCB− oocytes.

Conclusion(s): Brilliant cresyl blue–stained oocytes show more adenosine triphosphate content than BCB− oocytes. Results from IVF were affected by the oocyte quality while ICSI did not produce differences in embryo development or blastomere numbers. (Fertil Steril® 2012;■:■–■. ©2012 by American Society for Reproductive Medicine.)

Key Words: Oocyte quality, ICSI, IVF, embryos

I ntracytoplasmic sperm injection (ICSI) is the conventional assisted reproductive technology (ART) used in cases of severe male factor infertility, rather than in vitro fertilization (IVF). In cases of normospermia, total fertilization failure and low fertilization (defined as <25% fertilization) occur in 5%–15% and 20%, respectively, of the couples undergoing ART (1). Recurrent failure in ART could be explained by low oocyte quality expressed by the lack of sperm penetration, oocyte activation failure, and blockage of embryo development. In a clinical context, selecting good-quality oocytes and embryos is the key to improving ART. Whereas the nuclear maturation of the oocyte is easily identified by the appearance of the first polar body, cytoplasmic maturation, which is also essential for successful fertilization and early embryo development, remains difficult to measure. We have previously shown using sheep oocytes that brilliant cresyl blue (BCB) staining promotes the selection of the largest and most competent oocytes or development to the blastocyst stage with highest mitochondria and MPF activity (2). The stain is based on the ability of the BCB dye to be reduced by glucose-6-phosphate dehydrogenase (G6PDH) activity; thus, oocytes that have reached their growth phase and have low G6PDH activity cannot reduce BCB to a colorless compound exhibiting a blue-colored cytoplasm (BCB+) and the growing oocytes with high G6PDH activity are able to reduce the blue compound, which results in a colorless oocyte cytoplasm (BCB−).

In conclusion, BCB staining is a good system for selection of oocytes according to their competence.

Mitochondria are key players in cytoplasmic activity providing, among other biochemical factors, adenosine triphosphate (ATP) through oxidative phosphorylation and the citric acid cycle. Better-quality oocytes contain significantly higher levels of ATP and produce significantly higher blastocyst rates after fertilization (3–5).

The difficulty in acquiring human oocytes for research has hindered the development of reproductive technology. A valuable source of oocytes for research purposes are oocytes from animal donors. The aim of this study, therefore, is to use sheep as a model of good (BCB+) and low (BCB−) quality oocytes and to see how they react to ICSI and IVF procedures. In the current study, the use of sham-injected oocytes and parthenogenetically activated (PA) oocytes eliminates the influence of the sperm, and these procedures were also considered as a control for mechanical and chemical activation in selected BCB oocytes. Moreover, we analyze oocyte ATP production, pronuclear stage, blastocyst development, and inner cell mass and trophoectoderm cell number.

MATERIALS AND METHODS
Chemical
All chemicals were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA) unless otherwise specified.

Oocyte Collection
Ovaries from prepubertal ewes (3–6 months old) were obtained from a local abattoir and transported to the laboratory in sterile Dulbecco's phosphate-buffered saline (PBS) held at 34–37°C and were washed in PBS containing antibiotic-antimycotic (AB, GIBCO cat 14240-062). Because of the small follicle size of these ovaries, cumulus oocyte complexes were recovered by slicing the surface of the ovary in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid–buffered TCM-199 medium with 0.5 g/L fraction V bovine serum albumin (BSA). Oocytes with two or more complete layers of compact cumulus cells and homogenous cytoplasm were accepted for use.

Brilliant Cresyl Blue Test
Immediately after collection, morphologically selected cumulus oocyte complexes were washed three times in modified PBS (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 26 μM BCB diluted in mPBS for 1 hour 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 coloration: oocytes with blue cytoplasm or hypothetically grown oocytes (BCB+) and oocytes without blue cytoplasm coloration or hypothetically growing oocytes (BCB−).

In Vitro Maturation of Oocytes
Groups of 35–40 cumulus oocyte complexes of BCB+ and BCB− were washed and cultured for 24 hours at 38.5°C in a humidified air atmosphere with 5% CO_2 in 500 μL of maturation medium covered with mineral oil. Maturation medium consisted of TCM-199 supplemented with 5 μg/mL LH, 5 μg/mL FSH, 1 μg/mL 17β-E_2, 10 ng/mL epidermal growth factor, 0.2 mM sodium pyruvate, 2 mM L-glutamine, 100 μM cystamine, 10% (v/v) fetal bovine serum, and 2% (v/v) AB. After in vitro maturation, oocytes were fertilized in vitro, injected intracytoplasmically with sperm or not (without sperm), or activated parthenogenetically.

Adenosine-5′-triphosphate Analysis
Adenosine-5′-triphosphate analysis was performed using the ATP bioluminescent somatic cell assay kit (FLASC), per the manufacturer's protocol. Briefly, groups of 6 BCB+ and BCB− oocytes before (0 hours) and after (24 hours) in vitro maturation were completely denuded by gently pipetting and placed into an Eppendorf tube with 200 μL ultrapure water and stored at −80°C until use.

For reaction analysis, 0.1 mL of ATP assay mix working solution was added to a reaction vial for 3 minutes to hydrolyze any endogenous ATP. Then 0.05 mL of ultrapure water and 0.05 mL of sample were added to a separate vial containing 0.1 mL of 1x somatic cell–releasing reagent. Finally 0.1 mL of this solution was transferred to the reaction vial and the light emitted was immediately measured with a luminometer.

Intracytoplasmic Sperm and Sham Injection
Intracytoplasmic sperm injection was performed using morphologically selected thawed sperm in microdroplets of 10 μL of SOF media. Brilliant cresyl blue–stained and unstained metaphase II (MII) oocytes were injected at the 3 o'clock position with one spermatozoon with a minimum volume of medium (<5 pL PVP). Sham injections were performed as in ICSI excluding the spermatozoa and expelling a similar volume of PVP into the ooplasm. Injected oocytes were immediately activated in mPBS containing 5 μmol/L ionomycin (I24222; Invitrogen) for 4 min. Oocytes were washed and cultured in vitro.

In Vitro Fertilization
Highly motile spermatozoa from thawed semen were selected by using commercial Ovipure density gradient (Nidacon EVB S.L.) and fertilized with 1×10^6 spermatozoa/mL. Matured oocytes were partially denuded by gentle pipetting and transferred into fertilization medium consisting of synthetic oviductal fluid (SOF) (6). Fertilization was performed in 50-μL droplets of SOF medium supplemented with 20% of estrous sheep serum with a maximum of 15 oocytes per drop and incubated for 20 hours at 38.5°C in a humidified atmosphere containing 5% CO_2 and 5% O_2.

Parthenogenetic Activation
Matured oocytes were denuded by gently pipetting and activated in mPBS containing 5 μmol/L ionomycin for 4 minutes. After this initial activation, oocytes were clearly washed and

FIGURE 1
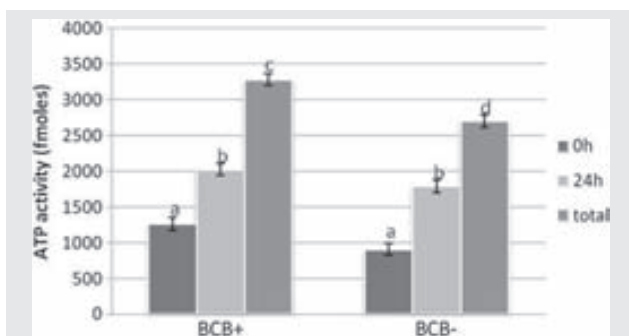
Adenosine triphosphate content in BCB+ and BCB− sheep oocytes before (0 hours) and after (24 hours) in vitro maturation. Different letters differ significantly (mixed procedure of SAS).

Catalá. Blastocyst production, after IVF and ICSI. Fertil Steril 2012.

placed in TCM-199 containing 1.9 mmol/L DMAP for 3 hours. After PA, presumptive zygotes were cultured in vitro.

In Vitro Culture

Intracytoplasmic sperm injection, IVF, PA, and sham presumptive zygotes were completely denuded by gently pipetting and cultured in groups of 6 zygotes for 7 days in 20 μL culture drops of SOF medium supplemented with 10% (v/v) fetal bovine serum at the same atmosphere conditions. Cleavage was evaluated after 24 hours of fertilization and blastocyst development at day 7 of in vitro culture.

Assessment of Pronuclear Formation

At 17 h after initial ionomycin exposure or coincubation with semen, presumptive zygotes were fixed in acetic acid-ethanol (3:1) and stained with 1% lacmoid to evaluate the nuclear stage. Zygotes were categorized as normally fertilized if one female and one male pronuclei were formed (2 PN). Zygotes with 3 PN were considered as polyspermic, zygotes with 1 PN or 1 PN and visible sperm head were considered activated.

Injected sham oocytes were considered activated when one or more pronuclei were observed.

Blastocyst Differential Staining

Blastocyst differential stain protocol was adopted from Thouas (7) and modified. Seven-day-old BCB+ and BCB− blastocyst were first incubated for approximately 15 seconds or until trophectoderm visibly changed color in solution with 1% Triton X-100 and 100 μg/mL propidium iodide. They were immediately transferred to a solution with ethanol and Hoechst 33258 for 1 hour. Stained blastocysts were transferred to a glass with a drop of glycerol, flattened with a cover slip, and then the cells were counted under a microscope. An Olympus BX50 was used with an ultraviolet lamp and excitation filter of 460 nm for blue and red fluorescence. The intense pink color represented the chromatin in nuclei of permeabilized trophectoderm cells that are stained both red (propidium iodide) and blue (Hoechst). Inner cell mass nuclei remained blue, because these cells had not been permeabilized.

Statistical Analysis

For the statistical analysis all the repetitions that were done were included in the analysis. Data was analyzed by using the mixed procedure of SAS (version 9.1; SAS Institute Inc.). For ATP, the model included the effects of treatment, number of hours, and their interaction. Although for PN, the model included only the treatment effect, and a square root–arcsine transformation was applied, but means are presented as back-transformed. Results are reported as least square means and residual errors of the means. For embryo development analysis, one-way ANOVA with Tukey's multiple comparison posttest was performed and for cell counting Fisher's exact test was performed (GraphPad Software, San Diego, CA, USA).

RESULTS

The results of the ATP content (fmoles) analysis is shown in Figure 1. Our findings indicate that selected BCB+ (3286.9 fmoles) oocytes produced more ATP than BCB− (2701.4 fmoles) oocytes. In addition, the ATP content in the oocytes

TABLE 1

Nuclear stage of BCB+ and BCB− oocytes after 17 hours of ICSI, IVF, PA, and sham procedures in sheep.

	ICSI		IVF		PA		Sham		*P* value	SEM
	BCB+	BCB−	BCB+	BCB−	BCB+	BCB−	BCB+	BCB−		
VG	0	0	1.45	3.33	3.52	5.00	0	0	.179	1.65
MII	10.14	9.22	14.95	12.90	20.18	31.51	30.99	18.33	.340	7.92
PN1	0[a]	0[a]	1.45[a]	0[a]	30.93[b]	29.28[b]	52.10[c]	34.00[b]	.0001	5.09
PN2	43.20[a]	37.58[a]	57.61[a]	42.73[a]	31.30[a]	23.07[a,b]	11.33[b]	29.67[a,b]	.002	8.76
PN3	0[a]	1.82[a]	24.54[b]	41.04[d]	14.07[b,c]	11.14[b,c]	5.58[a,c]	18.00[b,c]	.0002	4.94
MII-H	2.22	1.82	0	0	0	0	0	0	.758	1.34
PN1-H	44.44[a]	47.57[a]	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]	.0001	5.34
PN3-H	0	2.00	0	0	0	0	0	0	.654	0.93
Oocytes analyzed	57	51	57	55	56	52	57	58		

Note: Values in the same row (a-c) with different letters differ significantly (mixed procedure of SAS). GV = germinal vesicle; MII = Metaphase II; PN1, PN2, and PN3 = one, two or three pronuclei; MII-H = Metaphase II oocytes with non-decondense sperm head; PN1-H, PN3-H = one or three pronuclei with a non-decondense sperm head; SEM = standard error of the mean.

Catalá. Blastocyst production, after IVF and ICSI. Fertil Steril 2012.

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TABLE 2

Embryo development of BCB+ and BCB− oocytes at 7 days after ICSI, IVF, PA, and sham procedures in sheep.

	No. of oocytes		Cleavage (%)		Blastocyst/cleavage (%)		Total oocytes	No. of blastocysts (%)
	BCB+	BCB−	BCB+	BCB−	BCB+	BCB−		
ICSI	154	136	118 ± 2.3 (76.6)[a]	108 ± 4.0 (79.4)[a]	22 ± 1.0 (18.6)[a]	16 ± 1.2 (14.8)	290	38 ± 2.2 (13.1)[a]
IVF	137	149	104 ± 6.7 (75.9)[a,A]	90 ± 2.9 (60.4)[b,B]	33 ± 3.7 (31.7)[b,A]	6 ± 0.5 (6.7)[B]	286	39 ± 4.2 (13.6)[a]
PA	182	115	161 ± 6.7 (88.5)[b,A]	80 ± 2.5 (69.6)[a,b,B]	33 ± 1.0 (20.5)[a,A]	7 ± 0.5 (8.8)[B]	297	40 ± 1.5 (13.5)[a]
Sham	86	72	42 ± 2.2 (41.9)[c,A]	48 ± 2.0 (63.3)[a,b,B]	(7.7)[a]	(5.3)	158	6 ± 0.7 (3.8)[b]

Note: Mean ± SE, unless otherwise noted. Values in the same column (a, b, c) and row (A, B) with different letters differ significantly (Fisher test, $P < .05$).

Catalá. Blastocyst production, after IVF and ICSI. Fertil Steril 2012.

increases from 1,085 to 1,909 fmoles from the immature to the mature stage.

A total of 443 oocytes were analyzed for the nuclear stage after 17 hours of ICSI, IVF, PA, and sham procedures (Table 1). No differences in 2PN were found among groups. Polyspermic zygotes (PN3) were more numerous after IVF in BCB− (41.04%) than BCB+ (24.54%) oocytes.

Table 2 shows the results of blastocyst development of BCB selected sheep oocytes after different fertilization procedures. After ICSI, no differences in blastocyst percentage were found between BCB+ and BCB− oocytes (14.3% and 11.8%, respectively). However, the percentage of cleavage and blastocyst development was significantly different between BCB+ and BCB− oocytes after IVF and PA. Taking into account the total data, we did not find differences in blastocyst numbers between the ICSI, IVF, and PA procedures (13.1%, 13.6%, and 13.5%, respectively), whereas sham oocytes produced only 3.8% of blastocysts.

Table 3 shows the results of blastocyst quality assessed by the number of blastomeres in the trophectoderm and the inner cell mass of embryos coming from BCB selected oocytes after ICSI, IVF, PA, and sham injection. We did not find any difference in the blastocyst cell numbers between the different fertilization procedures or oocyte quality except for the IVF group, which had fewer blastomeres in BCB− than in BCB+ oocytes (43.9 and 69.7, respectively).

DISCUSSION

The aim of this study was to test the effect of oocyte quality, assessed by the BCB test, on blastocyst development after IVF and ICSI. Oocytes fertilized by ICSI did not show differences in blastocyst development between BCB+ and BCB− oocytes (14.3% and 11.8%, respectively). After IVF, good-quality oocytes (BCB+) developed up to the blastocyst stage at a higher percentage than BCB− oocytes (24.1% and 4.0%, respectively). However, this low percentage of blastocysts coming from BCB− oocytes was not due to a reduction in oocyte sperm penetration or a reduction of oocyte activation, as Table 1 shows. In addition, higher percentages of polyspermic oocytes were observed in BCB− oocytes compared with BCB+ oocytes. Oocytes of different quality undergoing PA showed a decrease in blastocyst percentages for BCB− oocytes (6.1%) compared with BCB+ oocytes (18.1%), suggesting an incomplete or abnormal cytoplasmic competency in these lower-quality oocytes. This behavior was similar to IVF; therefore, the PA technique could be suitable for studying oocyte quality. Several investigators have found higher blastocyst production in BCB+ compared with BCB− oocytes, as was reported in goats (8), sheep (2), cattle (9), buffalo (10), mice (11), pig (12), and horse (13). In contrast, no differences in blastocyst production were found between BCB selected oocytes when the ICSI procedure was used. A possible explanation of the better embryo development of BCB− oocytes after ICSI could be the accurate selection of metaphase II oocytes before sperm injection and the lack of polyspermic zygote formation. Westerlaken et al. (14) concluded that in patients with a history of low fertilization rates, the percentage of fertilization was higher after ICSI compared with IVF.

Blastocyst cell numbers is an important indicator of embryo quality. Several investigators have suggested that embryos with a high number of blastomeres are more likely

TABLE 3

Inner cell mass, trophectoderm, and total cell number of blastocyst at day 7 of in vitro culture of BCB sheep selected oocytes after ICSI, IVF, PA, and sham procedures.

	Total no. of cells		ICM		TE		% ICM/total cells		ICM-TE ratio		Total blastocyst	
	BCB+	BCB−	BCB+	BCB−	BCB+	BCB−	BCB+	BCB−	BCB+	BCB−	No. analyzed	No. of cells
ICSI	71.1 ± 9.7	54.3 ± 5.8	18.6 ± 2.8	14.7 ± 2.6	52.6 ± 8.5	39.7 ± 3.7	26.1	27.0	1:2.8	1:2.7	13	62.7 ± 7.8
IVF	69.7 ± 6.2[A]	43.9 ± 3.4[B]	18.8 ± 1.8	12.8 ± 1.1	50.9 ± 5.1	31.1 ± 3.2	27.0	29.1	1:2.7	1:2.4	33	56.8 ± 4.8
PA	58.2 ± 6.3	49.0 ± 10.9	18.2 ± 1.9	14.0 ± 3.2	40.0 ± 6.6	35.0 ± 8.5	31.2	28.6	1:2.2	1:2.5	16	53.6 ± 8.6
Sham	60.7 ± 2.2	39.0 ± 10.7	19.0 ± 9.1	11.0 ± 4.9	41.7 ± 10.7	28.0 ± 8.6	31.3	28.0	1:2.2	1:2.6	8	49.9 ± 6.5

Note: Mean ± SE, unless otherwise noted. Values in the same row (A, B) with different letters differ significantly (Fisher test, $P < .05$). ICM = inner cell mass; TE = trophectoderm.

Catalá. Blastocyst production, after IVF and ICSI. Fertil Steril 2012.

to implant and give rise to live offspring (15–17). In our study, we did not find differences in cell numbers among the ICSI, IVF, PA, and sham procedures, suggesting there are no differences in embryo quality according to the technique used. Westerlaken et al. (14) found no significant differences in pregnancy rate between transfers of ICSI embryos and transfers of a combination of one ICSI and one IVF embryo, concluding that there is no difference in embryo quality between the two procedures after embryo transfer. Our results showed that IVF BCB+ oocytes produced blastocysts with higher total cell numbers than those produced from BCB− oocytes. Similar results were found in cows after IVF (18) and nuclear transfer (9).

In our study, we found higher levels of ATP in BCB+ than in BCB− oocytes. Our findings also indicate that the ATP content increases during in vitro maturation as was previously reported in cows (5). The developmental potential of the embryo and the outcome of IVF have been shown to be related to both ATP and mitochondrial content in human oocytes (3). Santos (19) showed an increase in mitochondrial DNA (mtDNA) copy number in fertilized oocytes (250,454 copies) with respect to unfertilized oocytes (163,698 copies). We have previously found similar results (2) in which BCB+ oocytes contain more active mitochondria than BCB− oocytes. These results lead us to suggest that BCB staining is a good indicator of oocyte quality as observed in the ATP content and IVF embryos.

In conclusion, oocyte quality, assessed by BCB staining, significantly affects the percentage and quality of the blastocysts obtained after IVF. However, this effect was not observed in blastocysts from sheep oocytes after ICSI.

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General discussion

Oocytes recovered from ovaries of prepubertal and adult animals in sheep (O'Brien et al. 1996; O'Brien et al. 1997) and cattle (Revel et al. 1995) showed that although meiotic maturation, fertilization and cleavage rates did not differ between oocyte donor ages, the blastocyst formation rates were significantly lower in prepubertal oocytes than in adult oocytes. Armstrong (2001) concluded that oocytes coming from prepubertal donors have a lower quality versus oocytes from adult donors. In our first study using prepubertal sheep oocytes (Catalá et al. 2011) we produced an average of 13% of blastocyst from the total oocytes fertilized, similar to O'Brien's *et al.* (1997) 12.6% of blastocyst with prepubertal donor. Using BCB test, oocytes stained with 13 µM or 26 µM BCB showed an increase of blastocyst to 21% and 17%, respectively for the BCB+ group compared to 9% and 5% of blastocyst for the BCB- group. In this first approach using the BCB stain, the results obtained were positive and good prospects for continuing using this technique.

Previous studies in our laboratory using prepubertal goat oocytes showed that not only the age of the oocyte donor is crucial for embryo production, but also the size of oocyte plays an important role in embryo production. Goat oocytes with a diameter bigger than 125 µm showed significant higher MPF activity and blastocyst cell number after IVF (Anguita et al. 2007) and ICSI (Jimenez-Macedo et al. 2007).

The BCB test is a non invasive methodology that allows the selection of oocytes with bigger diameter among a heterogeneous pool. The BCB compound is used to determine the intracellular activity of G6PDH. The activity of this enzyme gradually decreases as oocytes reach their growth phase (Mangia and Epstein. 1975). BCB dye can be reduced by the G6PDH enzyme activity, thus oocytes that have reached their growth phase cannot reduce BCB to a colorless compound exhibiting a blue colored cytoplasm (BCB+). In our work with prepubertal sheep oocytes, BCB differentiate two populations of oocytes: the BCB+ oocytes with a diameter media of 123.66 µm producing significantly more blastocysts than oocytes BCB- with a diameter of 106.62 µm (Catalá et al. 2011). To our knowledge this was the first time that the BCB test was used in sheep. The BCB has been previously used in other species at different concentrations, 13 µM BCB was the best choice in selecting pig oocytes (Egerszegi et al. 2010) and 26 µM for cow (Pujol et al. 2004; Bhojwani et al. 2007) and goat (Rodriguez-Gonzalez et al. 2002). In our laboratory, with our pool of oocytes and under our conditions, both concentrations showed good results in differentiating the two population of oocytes, that is why we used 13 µM in the first and second work and in the third work we have changed to 26 µM BCB. The differences in using these 2 concentrations is that 13 µM seemed to be slightly more selective but only 19% were BCB+ oocytes, while 26 µM BCB appears to be slightly less

selective but a significant larger number of BCB+ oocytes (28%) were recovered (Catalá et al. 2011).

In our study, in which the BCB stain showed a good efficiency in separating the two different populations of oocytes; we analyzed the active mitochondria, the maturation promoting factor, the adenosine tri-phosphate and the mRNA expression of special genes in order to determine differences in their cytoplasm.

Mitochondria are maternally inherited organelles and are important to the cell because they supply the ATP needed for almost all processes (Stojkovic et al. 2001). Studying mitochondrial activity in relation with oocyte quality we found that at germinal vesicle (GV) stage there were no differences in mitochondrial activity between BCB+ and BCB- groups (2834 ± 223.42 and 3519 ± 288.48 arbitrary units, respectively). However after IVM, BCB- oocytes showed a significant reduction (1565 ± 113.8 and arbitrary units) of mitochondrial activity while BCB+ remained constant (Catalá et al. 2011). This fact could indicate a positive relationship between mitochondria activity at MII stage and embryo development. Other publications showed different responses of the mitochondria. In cow, Torner *et al.* (2008) observed higher mitochondrial activity in BCB- oocytes than in BCB+, speculating that low quality oocytes need more ATP for unfinished processes. Closer to our results, in pig Egerszegi *et al.* (2010) found higher mitochondrial activity in the BCB+ group compared to the BCB- oocytes but at GV stage and after IVM, no differences were found between either group of oocytes. They attributed this to the fact that BCB+ oocytes increased their respiratory activity to provide ATP for energy-demanding processes of the GVBD and the subsequent condensation of chromatin up to MII stage, while BCB- showed no changes in mitochondrial activity during meiosis and only a few of them reached MII stage.

When analyzing the ATP content we have found higher levels of ATP in BCB+ than in BCB- oocytes. Our findings also indicate that the ATP content increases during IVM as was previously reported in cow (Stojkovic et al. 2001). In human, the IVF outcomes and the developmental potential of the embryo seemed to be related to the ATP and mitochondrial content (Van Blerkom et al. 1995). Under our conditions, in prepubertal sheep oocytes there exists a positive relation between the oocyte quality, the mitochondrial activity and ATP content in the cytoplasm.

As we have previously said, meiosis is regulated by the activity of the MPF. In our study, after IVM, significantly higher MPF activity was found in good quality than in low quality oocytes. Salamone *et al.* (2001) comparing calf and cow oocytes found that following IVM the activity of MPF was substantially lower in calf oocytes than in oocytes of adult cattle. Similar results

were found by Ledda *et al.*(2001) who compared adult and prepubertal sheep oocytes as a model of high and low competent oocytes, showing that after IVM, prepubertal oocytes evidenced a lower MPF activity than adult oocytes and this could be due to morphological anomalies. Bogliolo *et al* (2004) showed a higher MPF activity of *in vivo* matured oocytes than *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 up 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).

We can say that staining prepubertal sheep oocytes with 13 μM or 26 μM BCB during 1 hour seemed to be a good selector of more competent oocytes that have more active mitochondria, ATP content and MPF activity and produce significantly more blastocyst percentage after IVF. Subsequent to our study (Catalá et al. 2011), Mohammadi *et al.*(2011a) stained adult sheep oocytes with 26 μM BCB during 90 min, where 55% of them were BCB+, showing higher diameter and blastocyst production (147 μm, 34%) than BCB- (130 μm, 4%) group. He also concluded that the BCB test is a good method to select oocytes with high diameter and embryo competence.

Wu's et al (2006), using prepubertal pig oocytes concluded that oocytes coming from small follicles and cultured in a growth media supplemented with ITS and AA previously to the conventional maturation media, significantly improved oocyte competence and embryo development. In our work, using the growth media (adapted to the sheep protocol) and a variation of it during 12 or 24 hours of the IVM in BCB- oocytes, no differences were found between BCB- matured in conventional IVM media and BCB- oocytes matured in the different combinations of the growth media. In sheep oocytes, Mohammadi-Sangcheshmeh *et al.* (2012) observed that growing oocytes (unstained BCB oocytes) not only develop a lower rate of blastocysts but also the percentage of oocytes reaching the metaphase II after IVM was significantly reduced. The absence of effect of the media could be due to that unstained oocytes or growing oocytes are in a earlier stage of an extreme lack of cytoplasmic maturation of the prepubertal sheep oocytes making this low quality oocytes non recoverable by this *in vitro* growth medium.

ICSI is an ART technology that produces embryos under *in vitro* conditions; especially it is used in human reproduction to solve not only problems related to male infertility but also problems related with low oocyte quality (Saito et al. 2000). In our case, prepubertal sheep oocytes showed to have a high number of cytoplasmic incompetent oocytes with low mitochondria activity, MPF and ATP content that could not be saved by the growth maturation

media. That is why we considerate that these kind of deficient oocytes are suitable to undergo ICSI.

In our study when performing ICSI we achieved similar blastocyst percentages between BCB+ and BCB- (14.3% vs. 11.8%, respectively) whereas with IVF (24.1% vs. 4.0%, respectively) and PA (18.1% vs. 6.1 %, respectively) the difference between good and low quality oocytes continued to be visible (Catalá et al. in press). A possible explanation of this good embryo development of BCB- oocytes after ICSI could be the accurate selection of MII oocytes carried out before sperm injection and the lack of polyspermic zygotes formed. This was in line with Saito's *et al*. (2000) results in human oocytes, in which they concluded that poor oocyte quality are a good indication for the use of ICSI, exposing the same hypothesis as ours.

According to the results obtained in these 3 studies, the BCB stain is a good test to select more competent sheep oocytes for in vitro embryo production. Stained BCB oocytes also showed higher mitochondrial activity, ATP content and MPF activity than unstained oocytes. None of the mRNA studied in this work have shown a correlation with the oocyte quality. Unstained oocytes have been unable to improve their embryo development after being cultured in Growth Media which means that these oocytes are in a growing stage and not competent to develop up to blastocyst

Finally we can conclude that the BCB test is an easy, fast and suitable methodology to select the best quality oocytes from a large and heterogeneous pool of oocytes. In experiments carried out with oocytes coming from ovaries recovered from a slaughterhouse the BCB test is a useful method to be incorporated in the protocol of in vitro embryo production using in vitro fertilization. However, the BCB test is less interesting when working with a small number of oocytes such as in Laparoscopic Ovum Pick Up (LOPU) or ICSI.

Conclusions

1- The BCB stain is a useful methodology to select from a large and heterogeneous pool of oocytes, the largest and most competent sheep oocytes for blastocyst production after IVF.

2- Oocyte stained by BCB show higher mitochondrial activity, higher MPF activity and more ATP content than unstained oocytes.

3- Unstained BCB oocytes did not improve their blastocyst production after being exposed to a Growth Media.

4- After IVF, selected BCB oocytes develop up to blastocyst stage in significantly higher percentage than unstained BCB oocytes. However, ICSI improve blastocyst production in unstained oocytes without differences to BCB selected oocytes.

Chapter 9

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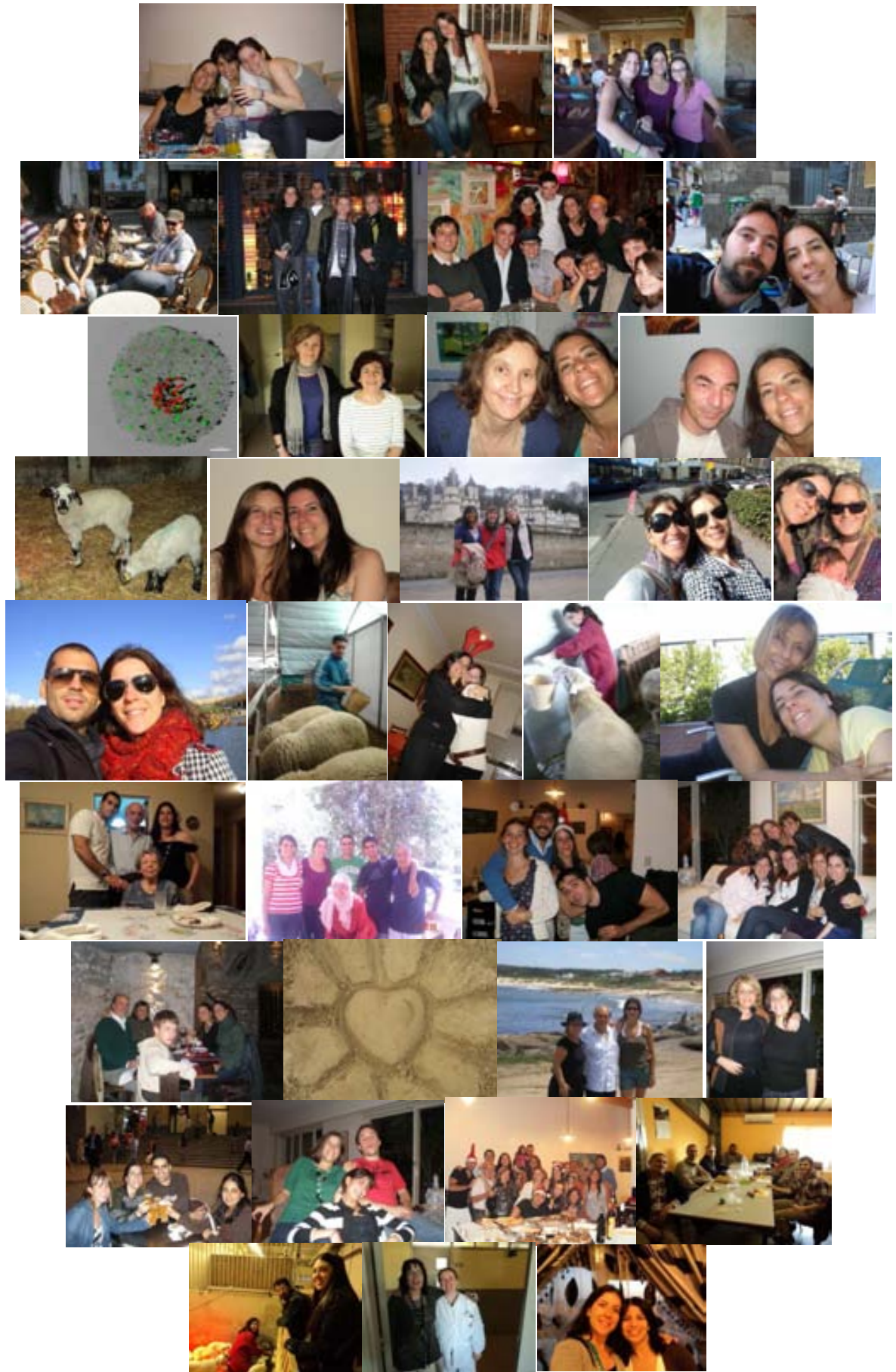
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3/09/09: Después de horas hablando del BCB con María, Dolors y Roser, Montse concluye, "Pero…. El BCB que es?"-