Resultados

SELECTION OF PREPUBERTAL GOAT OOCYTES USING THE BRILLIANT CRESYL BLUE TEST

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ABSTRACT

Brilliant Cresyl Blue stain allows determining the activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme synthesized in growing oocytes but with a decreased activity in oocytes that have finished their growth phase. The objective of this study was to evaluate the utility of the Brilliant Cresyl Blue (BCB) test as an indirect measure of oocyte growth, in order to select competent prepubertal goat oocytes for *in vitro* embryo production. Oocytes were exposed to BCB diluted in PBS and were classified according to their cytoplasm coloration: BCB+ (oocytes with a blue cytoplasm or grown oocytes) and BCB- (oocytes without a blue cytoplasm or growing oocytes). After exposure to different BCB concentrations, *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and embryo development parameters were evaluated. Matured oocytes were considered those oocytes that reached the metaphase II stage after being cultured for 27 h. Oocytes showing 2 pronuclei at 20 h post-insemination were classified as normally fertilized oocytes. Embryo development was assessed 8 days post-insemination and the percentage of total embryos, morulae and blastocysts recorded. The mean percentage of BCB+ oocytes was 29.4 %. Mean diameter of BCB+ oocytes (136.6 ± 6.3 μm) was higher ($P<0.001$) than that of BCB- oocytes (125.5 ± 10.2 μm). The percentage of BCB+ oocytes reaching the MII stage (81.4%) was higher ($P<0.05$) than those of BCB- (52.5%) and control oocytes (72.4%). Normal fertilization rate of BCB+ oocytes was also higher (23.5%) than those of BCB- (8.2%; $P<0.0001$) and control oocytes (11.9%; $P<0.05$). The percentages of total embryos undergoing development to $\geq$ 8-cell and the morula plus blastocyst stages were higher ($P<0.05$) in the group of BCB+ (41.3% and 12.0%, respectively) than BCB- oocytes (21.3% and 3.6%, respectively). In conclusion, the BCB test is a useful method for the selection of more competent immature prepubertal goat oocytes for *in vitro* embryo production.

Key words: Brilliant cresyl blue, oocytes, IVF, prepubertal, goat

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INTRODUCTION

The use of juvenile animals in embryo transfer programs would allow improving the genetic gain by reducing the generation interval (21, 35). Oocytes obtained from prepubertal animals can produce viable offspring after *in vitro* maturation, fertilization and embryo culture (13, 15, 24, 28, 30,). However, the developmental capacity of the embryos derived from prepubertal animals is lower compared to that of embryos produced from adult animals in different species, including cow (6, 13, 14, 20, 30, 32), sheep (1, 17, 18, 24, 25) and pig (27). In goats, a lack of development up to the blastocyst stage in prepubertal animals has been reported (12, 16). These observations could indicate an incomplete maturation (30) or cytoplasmic deficiencies of the oocytes obtained from prepubertal animals, which results in low capacity to support embryo development *in vitro* (8).

Oocytes recovered from ovaries obtained from slaughtered animals are heterogeneous, coming from follicles in different stages of growth and atresia. In goats, Crozet *et al*. (3, 4, 5) found a direct positive relationship between follicular diameter, oocyte diameter, meiotic competence and embryo development, showing that 56% and 96% of oocytes were able to reach the MII stage after IVM when oocytes were recovered from follicles of 2-3 mm and larger than 3 mm, respectively. Moreover, they showed that the goat blastocyst production was 6% with oocytes from follicles of 2-3 mm, 12% from follicles ranging from 3.1 to 5 mm, 26% from follicles larger than 5 mm and 41% with ovulated oocytes. With prepubertal goats, Martino *et al* (23) compared different oocyte collection techniques (dissection, aspiration and slicing), in order to obtain the largest number of selected oocytes for IVM-IVF programs, and concluded that slicing is the most useful technique. However, this technique does not allow selection by follicular diameter.

Brilliant Cresyl Blue (BCB) stain allows us to determine vitally the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme synthesized in the first half of the S phase, during the oocyte growth phase (39). G6PD activity plays a critical role in cell growth by providing NADPH for redox regulation (36). BCB is a blue compound reduced to a colorless one by G6PD activity. Oocytes that have finished their growth phase show a decreased G6PD activity. These oocytes exhibit a cytoplasm with a blue coloration because they do not reduce BCB to a colorless one. Increased G6PD enzymatic activity has been related to deleterious effects on maturation and fertilization of porcine oocytes (9). The BCB test has been successfully used to select pig oocytes for *in vitro* maturation and fertilization (9), and to improve the performance of the homologous *in vitro* penetration (hIVP) assay by selecting immature oocytes for assessing the penetrating ability of boar spermatozoa (31). In heifers, the BCB test has been used to select more competent oocytes for *in vitro* embryo production (29).

The purpose of this study was to evaluate the BCB test as an indirect measure of oocyte growth in order to select more competent prepubertal goat oocytes for *in vitro* maturation, *in vitro* fertilization and subsequent embryo culture.

MATERIALS AND METHODS

Oocyte Collection

Ovaries from prepubertal goats, approximately 2 months old, were obtained from a local slaughterhouse and transported at 37ºC in Dulbecco's phosphate-buffered saline (PBS, Sigma, P-4417) containing 50 μg/mL gentamycin. Within 2 hours of slaughter, the ovaries were washed three times in PBS containing gentamycin. Follicular contents were recovered by slicing the ovaries with a surgical blade in a 60 mm culture dish containing TCM199 (Sigma, B-2520), supplemented with 135 μg/mL NaHCO3, 11.1 μg/mL heparin-sodium salt (170 USP/mg; Sigma, 3393), 2% (v/v) steer serum (Donor Bovine Serum®, CanSera, Ontario, Canada) and 50 μg/mL gentamycin. Only oocytes with one or more complete layers of unexpanded cumulus cells and an evenly granulated cytoplasm were used.

Brilliant cresyl blue test

Immediately after oocyte collection, oocytes were washed 3 times in modified Phosphate Buffered Saline (mPBS), consisting in Dulbecco's PBS (Sigma, D-5773) modified by the addition of 1090 mg/L glucose, 35.2 mg/mL pyruvate, 0.4% (w/v) BSA (Sigma, A-9647, fraction V) and 50 μg/mL gentamycin. Oocytes were then exposed to BCB (Sigma, B-5388) diluted in mPBS for 90 min at 38.5ºC in a humidified air atmosphere. After exposure to different BCB concentrations (13, 26, 39 or 52 ìM BCB in mPBS), the oocytes were washed 3 times in mPBS and classified into 2 groups depending on their cytoplasm coloration: BCB+ and BCB-. BCB+ oocytes showed blue cytoplasm coloration. BCB- oocytes reduce BCB to a colorless compound, showing their cytoplasm without blue coloration.

In vitro Maturation of Oocytes

After oocyte classification, oocytes were washed 3 times in the maturation medium. The maturation medium was TCM199 (Sigma, M-7528) supplemented with 2750 μg/mL sodium pyruvate, 146 μg/mL L-glutamine, 10% (v/v) steer serum, 10 μg/mL caprine-LH (supplied by Dr. J.F. Beckers, IRSIA Research Unit, University of Liege, Belgium), 10 μg /mL o-FSH (Ovagen®, Immuno Chemicals Products Ltd., Auckland, New Zealand), 1 μg/mL 17β estradiol (Sigma, E-2257) and 50 μg/mL gentamycin. Groups of 20-25 cumulus enclosed oocytes were transferred to 100-μl microdrops of maturation medium and incubated for 27 h at 38.5ºC in a humidified air atmosphere of 5% CO2 under mineral oil (Sigma, M-3516).

Sperm Preparation

At the end of the maturation period, oocytes were inseminated with fresh semen. Ejaculates from 2 Malagueño Bucks of proven fertility were collected into artificial vagina and transported within 30 min to the laboratory at 37ºC. Motility of sperm cells was evaluated under an inverted microscope and motile sperm fraction was separated by swim-up: 70 μL of semen were placed in each of several conical tubes under 2 mL Defined Medium (2) modified by Younis *et al*. (40) and referred to as mDM here, and incubated for 45-60

min in a humidified atmosphere of 5% CO_2 in air at 38.5ºC. After incubation, 600 µL from the top of each tube was removed and pooled in a sterile 15-mL centrifuge tube and centrifuged at 200 x g for 10 min. After discarding the supernatant, the resulting sperm pellet was resuspended 1:1 with mDM medium containing heparin (100 µg/mL heparin-sodium salt; 170 USP/mg) and incubated for 45-60 min in a humidified air atmosphere of 5% CO_2 at 38.5ºC (final concentration: 84x106 sperm/mL, approximately)

In vitro Fertilization of Oocytes

After maturation, groups of 20-25 oocytes were transferred into 100-µl fertilization microdrops of modified Tyrode s medium (TALP), as described by Parrish *et al*. (26), supplemented with 1 µg/ml hypotaurine (Sigma, H-1384) under mineral oil. After capacitation, sperm concentration was assessed with a hemacytometer, and an aliquot (5 µl) of the sperm suspension was added to the fertilization microdrops (final concentration: 3.5x106 sperm/mL). Culture was performed for 24 h in a humidified air atmosphere of 5% CO_2 at 38.5ºC.

Evaluation of Oocytes after IVM and IVF

To evaluate the nuclear stage after maturation, a sample of oocytes was fixed at 27 h of IVM and stained with 1% lacmoid (Sigma, L-7512). Oocyte development was measured by the percentage of oocytes reaching the metaphase II (MII) stage.

To evaluate the pronuclear stage after 20 h of IVF a sample of oocytes was processed in the same way as the oocytes fixed after IVM. Oocytes with a sperm tail in the cytoplasm were considered to be fertilized and were classified in 1 of 3 groups: 2PN (female pronucleus, male pronucleus and sperm tail; normal fertilization), polyspermy (2 or more sperm tails in the cytoplasm with non-decondensed heads or two or more decondensed heads in the cytoplasm) and asynchrony with a non-decondensed sperm head (female pronucleus and a non-decondensed sperm head).

In vitro Embryo Culture

Following 24 h of sperm exposure, oocytes were washed in the culture medium with the aid of a fine pipette to separate oocytes from any sperm cells. Embryo culture was performed in groups of 20-25 embryos in 20-25-µl microdrops (1 µl culture medium/embryo) of Synthetic Oviductal Fluid (34) modified by Takahashi and First (33) in 35 mm culture dishes under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Presumptive embryos were maintained in culture for 7 days. The culture medium was not changed during this period. After 24 h in culture (that is, 48 h after insemination), 10% (v/v) steer serum was added to the microdrops (0.1 µl serum/embryo). At the end of the culture period, total cell number of embryos was assessed with fluorescence microscopy after Hoechst staining, and the percentage of total embryos, morulae (>16 cells) and blastocysts (>32 cells) recorded.

EXPERIMENTAL DESIGN

In Experiment 1, the effect of BCB concentration on oocyte selection and viability after IVM and IVF was analyzed. Four BCB concentrations were evaluated: 13, 26, 39 and 52 µM BCB in mPBS. After 90 min of BCB exposure, oocytes were classified as BCB+ or BCB-. The percentage of selected oocytes by the BCB test (BCB+) was recorded for each BCB concentration. After BCB classification, the oocytes were maintained undisrupted in the maturation medium for 27 h. Control oocytes were washed and placed in culture immediately after their collection and maintained undisrupted in the maturation medium for 27 h. After IVM, the percentage of oocytes reaching the MII stage for each BCB concentration was evaluated. To study the possible negative effects of BCB, *in vitro* matured oocytes were inseminated. Fertilization parameters were then evaluated. Based on the results obtained in Experiment 1, 26 µM was the BCB concentration used in Experiments 2 and 3.

Experiment 2 was conducted to determine if a higher percentage of the oocytes selected by the BCB test had completed their intraovarian growth phase as compared with the BCB- oocytes.

After COCs collection and morphological selection, COCs were classified in three categories depending on the number and compactness of the cumulus cell layers and the homogeneity of the oocyte cytoplasm, according to a modified classification of Le Guienne (19) for bovine COCs: Grade 1, Grade 2 and Grade 3 COCs. Grade 1 COCs were considered when more than 5 complete and compact cumulus cells layers and a homogeneous oocyte cytoplasm was present. Grade 2 COCs were considered complexes with a homogeneous oocyte cytoplasm and when the whole cumulus was not present but there were more than 5 cumulus cell layers or 3 to 5 complete cumulus layers. Grade 3 COCs were considered when a homogeneous oocyte cytoplasm was present and the number of cumulus cell layers was 2 or less, usually a single layer. Oocytes were exposed to 26 µM BCB for 90 min at 38.5ºC. The percentage of selected oocytes by the BCB test was recorded for each COCs category.

Oocyte diameter was also measured by COCs category and BCB classification. Oocytes were denuded from cumulus cells and measured using a micrometric ocular (Olympus Optical, Hamburg, Germany) under an inverted microscope (x 200; Olympus). Oocyte proper diameter (excluding the zona pellucida) was measured as the mean length of two perpendicular axes.

The utility of the BCB test to select oocytes with a higher developmental competence was analyzed in Experiment 3. Oocyte viability after BCB exposure was measured by (i) the percentage of oocytes reaching the MII stage after IVM, (ii) the percentage of normal fertilization after 20 h of IVF, (iii) the percentage of total embryos, morulae and blastocysts after 7 days of culture. Three groups of treatment were designed: BCB+, BCB-, and control. After BCB classification, the oocytes were maintained undisrupted in the maturation medium for 27 h. Control oocytes were put in culture after their collection and maintained undisrupted in the maturation medium for 27 h. Oocytes were then inseminated and cultured as indicated above.

Statistical Analysis

Differences among treatment groups were calculated by means of chi-square analysis or Fischer s exact test where appropriate. The overall chi-square was calculated and found significant before the Fischer s exact test was performed to detect differences between treatment groups. One-way ANOVA with Tukey s post-test was performed using GraphPad InStat (version 3.01 for Windows 95, GraphPad Software, San Diego, California, USA) to analyze differences among groups in oocyte diameter. Kruskal-Wallis test with Dunn's Multiple Comparisons post-test was performed to analyze differences among groups in embryo cell number. Differences with a probability value of 0.05 or less were considered significant.

RESULTS

EXPERIMENT 1: EFFECT OF BCB CONCENTRATION ON OOCYTE SELECTION, IVM AND IVF OF PREPUBERTAL GOAT OOCYTES

Table 1 shows the percentage of selected oocytes and the rates of nuclear maturation obtained after exposure to different BCB concentrations (13, 26, 39 and 52 µM BCB). The percentages of BCB+ oocytes obtained after staining with 26 µM (30.1%), 39µM (29.4%) and 52 µM BCB (32.8%) were higher (P<0.0001) than those obtained with 13 µM (1.7%).

BCB+ oocytes exhibited a higher (P<0.0001) maturation rate (mean rate: 79.1%; n=254) than BCB- (52.5%; n=703) and control oocytes (62.1%; n=235). There were no significant differences in the percentage of BCB+ oocytes reaching the MII stage among BCB concentrations. There were no significant differences in nuclear maturation between control and BCB exposed oocytes (BCB+ plus BCB-), independently of the BCB concentration.

Figure 1. BCB+ cumulus-oocyte complex

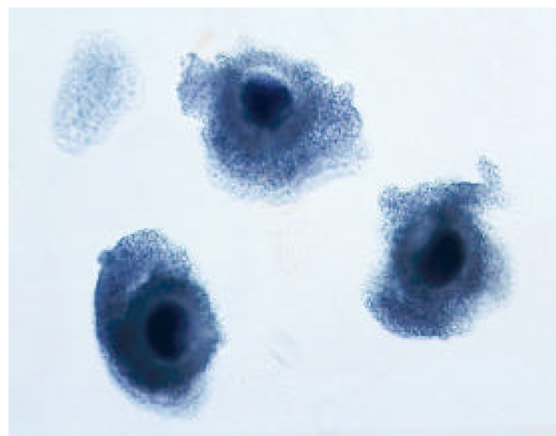

Figure 2 . BCB+ cumulus-oocyte complexes

Table 1. Effect of BCB concentration on selection and nuclear maturation after IVM of prepubertal goat oocytes (replicates = 10)

BCB concentr.	No.	Oocyte classification BCB+ (%)	BCB- (%)	BCB+ oocytes No.	MII (%)	BCB- oocytes No.	MII (%)	BCB+ plus BCB- No.	MII (%)
13 μM	303	5[b] (1.7)	298[a] (98.3)	3	3 (100)	247	141[a] (57.1)	250	144 (57.6)
26 μM	319	96[a] (30.1)	223[b] (69.9)	94	79 (84.0)	166	89[ab] (53.6)	260	168 (64.6)
39 μM	299	88[a] (29.4)	211[b] (70.6)	72	56 (77.8)	152	78[ab] (51.3)	224	134 (59.8)
52 μM	293	96[a] (32.8)	197[b] (67.2)	85	63 (74.1)	138	61[b] (44.2)	223	124 (55.6)
Control	-	-	-	-	-	-	-	235	146 (62.1)

[a,b]: values in the same column with different superscripts differ significantly ($P<0.05$).

Table 2 shows fertilization parameters of BCB+ oocytes after exposure to different BCB concentrations. BCB+ oocytes after exposure to 26 μM BCB showed a higher ($p<0.001$) percentage of fertilization (65.0%) than after exposure to 39 μM (33.3%) and 52 μM BCB (28.6%). There were no significant differences in normal fertilization (2PN) among BCB concentrations.

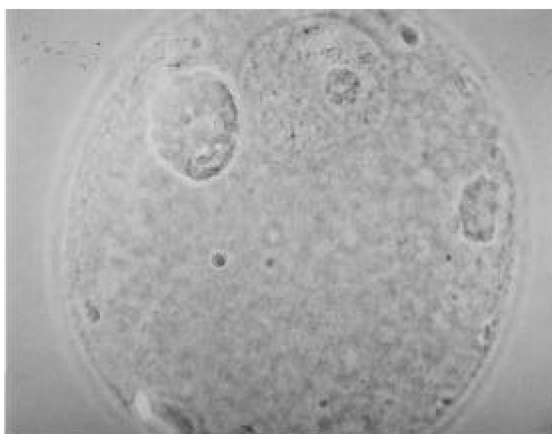

Figure 3. Immature oocytes (GV)

Figure 6. Mature oocyte (MII)

Table 2. Effect of BCB concentration on IVF of BCB+ prepubertal goat oocytes (replicates = 3)

BCB concentration	No. of BCB+ inseminated oocytes	No. of Fertilized oocytes (%)	No. of 2PN oocytes (%)
26 µM	60	39 (65.0)[a]	16 (26.7)
39 µM	39	13 (33.3)[b]	5 (12.8)
52 µM	42	12 (28.6)[b]	8 (19.0)
Control	85	46 (54.1)[a]	13 (15.3)

Fertilized oocytes: oocytes with a sperm tail in the cytoplasm; 2PN oocytes: oocytes with masculine and feminine pronuclei and one sperm tail.
a,b: values in the same column with different superscripts differ significantly ($P<0.05$).

EXPERIMENT 2: RELATIONSHIPS AMONG QUALITY OF CUMULUS OOCYTE COMPLEXES (COCS), OOCYTE DIAMETER AND OOCYTE SELECTION BY THE BCB TEST

Table 3 shows the percentage of oocytes selected by the BCB test after being classified according to COCs quality. Higher percentages of BCB+ oocytes were observed in Grade 1 and Grade 2 than in Grade 3 COCs ($P<0.05$). Also, a tendency ($P=0.06$) to a lower percentage of BCB+ oocytes was registered in Grade 2 in relation to Grade 1 COCs. In this experiment, the mean percentage of BCB+ oocytes was 32.1% (110/343).

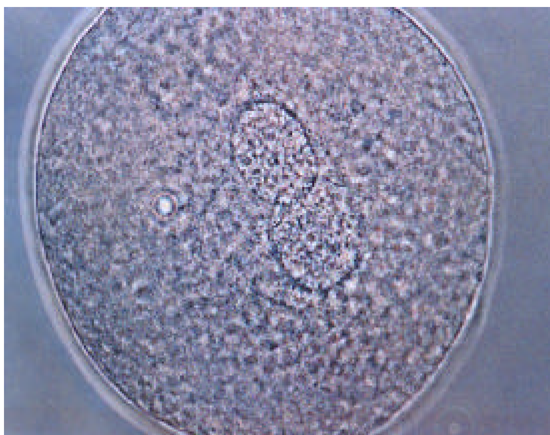

Figure 5. 2 PN oocyte (pronuclei in central position)

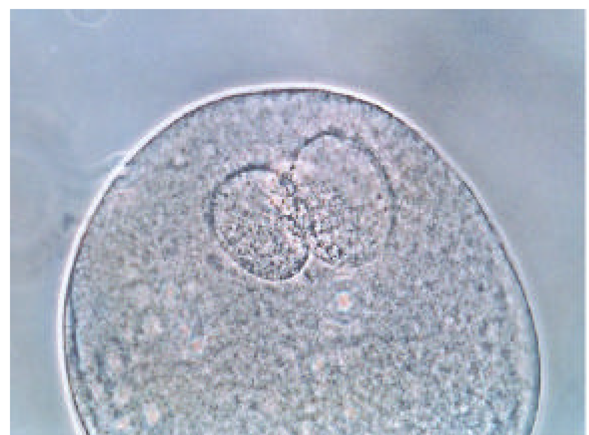

Figure 6. 2 PN oocyte

Table 3. Relationship between quality of cumulus oocyte complexes (COCs) and oocyte selection by the BCB test (replicates = 3)

COCs Quality	Total No. of COCs	Oocyte classification			
		BCB+		BCB-	
		No.	(%)	No.	(%)
Grade 1	97	48	(49.5)^{a}	49	(50.5)^{b}
Grade 2	97	34	(35.1)^{a}	63	(64.9)^{b}
Grade 3	149	28	(18.8)^{b}	121	(81.2)^{a}

a,b: values in the same column with different superscripts differ significantly (P<0.01).

Oocyte diameter of BCB+ and BCB- oocytes is shown in Table 4. The relationship between COCs quality and oocyte diameter is also shown in Table 4. The mean diameter of prepubertal goat oocytes obtained for *in vitro* embryo production was 129.3 ± 10.5 (mean ± SD). Overall, mean diameter of BCB+ oocytes (136.6 ± 6.3 μm) was higher (P<0.001) than that of BCB- oocytes (125.5 ± 10.2). In addition, BCB+ oocytes showed a larger size than BCB- oocytes for each COCs category (P<0.01). There were no significant differences among COCs grades in diameter of the oocytes selected by the BCB test (BCB+). Overall, oocytes from Grade 1 and Grade 2 COCs showed a larger size than those from Grade 3 COCs (p<0.01).

Table 4. Relationship between quality of cumulus oocyte complexes (COCs) and diameter of oocytes selected by the BCB test (replicates = 3)

Oocyte Classification	Total COCs		COCs Quality					
			Grade 1 COCs		Grade 2 COCs		Grade 3 COCs	
	No.	Diameter =	No.	Diameter =	No.	Diameter=	No.	Diameter=
BCB+	99	136.6 ± 6.3^{a}	42	136.8 ± 5.7^{a}	33	136.9 ± 5.3^{a}	24	135.8 ± 8.4^{a}
BCB-	188	125.5± 10.2^{b}	44	127.0 ± 9.2^{b}	50	128.8 ± 9.5^{b}	94	123.0 ± 10.4^{b}
TOTAL	287	129.3 ± 10.5	86	131.8 ± 9.1	83	132.0 ± 9.0	118	125.6 ± 11.3*

= Diameter: values in μm ± SD.
a,b: values in the same column with different superscripts differ significantly (P<0.01).
* Significantly different from other values within rows (P<0.05).

EXPERIMENT 3: SELECTION OF IMMATURE PREPUBERTAL GOAT OOCYTES BY THE BCB TEST FOR IVM-IVF-IVC

In this Experiment, the mean percentage of selected oocytes by the BCB test (BCB+) was 25.9%. Table 5 shows the nuclear stage of BCB exposed oocytes after 27 h of IVM. Most oocytes in all groups were capable of undergoing GVBD. However, a significant percentage of BCB- oocytes were blocked in their resumption of meiosis in the GVBD and, especially, in MI nuclear stages. The percentage of oocytes reaching the MII stage of BCB+ (81.4%) was higher (p<0.05) than those of BCB- (52.5%) and control oocytes (72.4%).

Table 5. Effect of the BCB test on nuclear stage of *in vitro* matured oocytes (replicates = 5)

Oocyte classification	Total No. of oocytes	GV No. (%)	GVBD No. (%)	MI No. (%)	An-Tel I No. (%)	MII No. (%)
BCB+	199	0 (0)	0 (0)^{b}	36 (18.1)^{b}	1 (0.5)	162 (81.4)^{a}
BCB-	777	8 (1.0)	45 (5.8)^{a}	314 (40.4)^{a}	2 (0.3)	408 (52.5)^{c}
Control	225	4 (1.8)	1 (0.4)^{b}	57 (25.3)^{b}	0 (0)	163 (72.4)^{b}

GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; An-Tel I: anaphase-telophase I; MII: metaphase II.
a,b,c: values in the same column with different superscripts differ significantly (P<0.05).

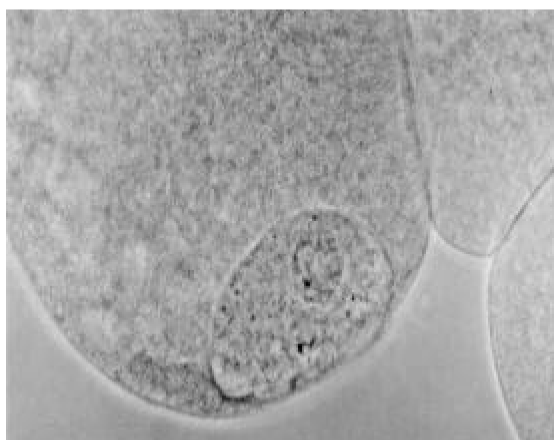

Figure 7. Oocyte in GV stage

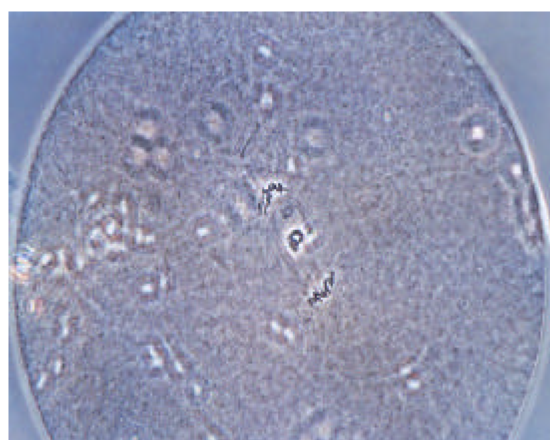

Figure 8. Oocytes in Anaphase-telophase stage

Table 6 shows the fertilization parameters of BCB exposed oocytes. There were no significant differences in fertilization rates among treatment groups. The percentage of normal fertilization (2PN) of BCB+ oocytes was higher (23.5%) than those of BCB- (8.2%; P<0.0001) and control oocytes (11.9%, P<0.05). There were no significant differences in polyspermy (range 45.3 to 49.8%) and asynchronous fertilization with a non-decondensed sperm head (range 8.8 to 16.4%) among treatment groups. The percentage of polyspermic oocytes was elevated in all groups of treatment, including the control.

Table 6. Effect of the BCB test on *in vitro* fertilization of *in vitro* matured oocytes (replicates = 5)

Oocyte classification	Total No. of inseminated oocytes	Fertilized oocytes			
		Total No. (%)	2PN No. (%)	Polyspermic No. (%)	Asynchronous No. (%)
BCB+	102	81 (79.4)	24 (23.5)[a]	48 (47.1)	9 (8.8)
BCB-	245	175 (71.4)	20 (8.2)[b]	122 (49.8)	33 (13.5)
Control	159	117 (73.6)	19 (11.9)[b]	72 (45.3)	26 (16.4)

2PN: 2 pronuclei + 1 sperm tail; Polyspermic: oocytes with 2 or more sperm tails in the cytoplasm with non-decondensed heads or two or more decondensed heads in the cytoplasm; Asynchronous: oocytes with a non-decondensed sperm head and 1 feminine pronucleus.
a,b: values in the same column with different superscripts differ significantly ($P<0.05$).

Table 7 shows the rates of cleavage and development to the blastocyst stage of oocytes exposed to the BCB test. The percentage of total embryos after culture did not differ among groups. Eight days after insemination, the percentage of embryos with 8 or more cells (41.3%) was higher ($P<0.001$) in the group of BCB+ oocytes in comparison to the BCB- (21.3%) and control groups (19.2%). The percentage of BCB+ oocytes that reached the morula and blastocyst stages (12.0%) was also higher ($P<0.05$) than that of BCB- oocytes (3.6%). Also, a tendency ($P=0.06$) to a higher percentage of blastocysts was observed in BCB+ oocytes (4.0%) in relation to BCB- oocytes (0.5%). There were no significant differences among treatments in the mean cell number of morulae plus blastocysts (BCB+: 34.1 ± 19.9 cells; BCB-: 28.8 ± 20.7 cells; control group: 21.5 ± 12.1 cells).

Table 7. Effect of the BCB test on embryo development of *in vitro* matured and fertilized prepubertal goat oocytes (replicates = 5)

Oocyte classification	Total No. of inseminated oocytes	Embryo development at day 8 postinsemination			
		Total embryos No. (%)	≥ 8 cell embryos No. (%)*	Morulae +blastoc. No. (%)*	Blastocysts No. (%)*
BCB+	119	75 (63.0)	31 (41.3)[a]	9 (12.0)[a]	3 (4.0)
BCB-	342	197 (57.6)	42 (21.3)[b]	7 (3.6)[b]	1 (0.5)
Control	126	78 (61.9)	15 (19.2)[b]	6 (7.7)[ab]	1 (1.3)

Blastoc: blastocysts; * Percentages calculated from total embryos.
a,b: values in the same column with different superscripts differ significantly ($P<0.05$).

DISCUSSION

Immature oocytes stained by the BCB test (BCB+) showed higher rates of maturation, normal fertilization and development to the morula and blastocyst stages than not stained oocytes (BCB-). This test measures the reduction of the BCB, a vital blue dye, to a colorless compound by glucose 6-phosphate dehydrogenase (G6PD) activity. Thus, oocytes that have finished their growth phase will show decreased G6PD activity and will exhibit blue coloration, because BCB will remain non-reduced in the cytoplasm. The absence of enzymatic activity of G6PD can be an indirect measure of fully-grown oocytes that have finished their intraovarian growth phase.

Previous reports using the BCB test in pig oocytes employed a concentration of 13 μM BCB (9, 31). However, in our study with prepubertal goat oocytes, this concentration yielded a very low percentage of BCB+ oocytes (1.7%). The concentration used in our study to apply the BCB test was 26 μM. This concentration allowed us to obtain a high rate of selected oocytes without apparent loss of viability because no differences were found in maturation and fertilization rates between the total numbers of oocytes exposed to 26 μM BCB and control oocytes, indicating the absence of toxic effects from the BCB test. Tiffin *et al*. (37) reported that BCB could be used effectively in the study of embryo metabolism without being lethal after exposure with 26 μM BCB. On the other hand, this BCB concentration has been used to select immature heifer oocytes for IVF, and a higher embryo development in BCB+ group compared with BCB- and control groups was reported in a previous study (29). Nevertheless, the percentage of BCB+ oocytes obtained in the present study, employing 26 μM BCB, was still lower (29.4%) than those reported in pigs, (91.2%; Ericsson *et al*. (9); 63.3 to 80.6%; Roca *et al*. (31)) and heifers (62.4%; Pujol *et al*. (29)). We can deduce that only a low proportion of prepubertal goat oocytes, collected by slicing and selected on the basis of visual assessment of morphological features, such as homogeneity of the cytoplasm and number of cumulus layers, have completed their growth.

Oocyte diameter is a determinant factor in acquiring meiotic competence (11). In adult goats, Crozet *et al*. (5) described the relationship between oocyte diameter and follicle diameter, thus oocytes with a mean diameter of 96 μm are recovered from follicles smaller than 0.5 mm, oocytes of 120 μm from follicles of 0.5-0.8 mm, oocytes of 125 μm from follicles of 1-1.8 mm, oocytes of 136 μm (range 125-146 μm) from follicles of 2-3 mm and follicles larger than 3 mm have oocytes ranging from 130 to 146 μm. The MII stage was reached in 86% of adult (7) and 72% of prepubertal goat oocytes (22) recovered from follicles larger than 3 mm. In our study, the mean diameter of prepubertal goat oocytes was 129.3 μm, 136.6 μm for BCB+ and 125.5 μm for BCB- oocytes. The percentage of metaphase II oocytes after IVM of BCB+ oocytes (81.4%) was higher than that of BCB- (52.5%) and control oocytes (72.4%). This finding confirms that, to a great extent, BCB+ oocytes have finished their growth phase when they are directly recovered from the ovary. Also, in pig oocytes selected as blue after staining with the BCB test, the oocytes were significantly larger (113.1 μm) than colorless oocytes (100.3 μm) (31). We suggest that the BCB test is a useful technique to select prepubertal goat oocytes.

The quality of the oocytes, measured according to the quality and aspect of the cumulus oophorus, increases as the follicular diameter increases (22). In our study, the percentage of selected oocytes by the

BCB test was higher in Grade 1 and Grade 2 than in Grade 3 COCs. However, only 49.5% of the Grade 1 and 35.1% of Grade 2 COCs had BCB+ oocytes. Normally, Grade 1 and Grade 2 COCs are morphologically selected for *in vitro* embryo production programs. Our results show that selection of prepubertal goat oocytes, based only on morphological criteria, will include oocytes in different stages of growth. Oocytes that appear morphologically similar to each other can have quite different developmental capabilities post-insemination; therefore morphology is not always a suitable indicator of oocyte selection.

The data presented here show a low blastocyst formation rate. Previous experiments also reported a lack of development up to the blastocyst stage in prepubertal goats: Izquierdo *et al*. (12) using oocytes from 2 months-old females obtained in a slaughterhouse (10% blastocysts) and Koeman *et al*. (16) with oocytes collected by laparoscopic folliculocentesis from 2-5 month-old hormonally stimulated goats (8% blastocysts). Crozet *et al*. (3) suggest that only a small proportion of the oocytes recovered from follicles larger than 2 mm in diameter can support embryonic development because the capacity to complete cytoplasmic maturation develops beyond the acquisition of meiotic competence. Thus, they showed that the goat blastocyst production was 6% with oocytes from follicles of 2-3 mm, 12% from follicles ranging from 3.1 to 5 mm, 26% from follicles larger than 5 mm and 41% with ovulated oocytes. In prepubertal goats, the number of 3-6 mm follicles per ovary is 1.1 (23) that means that follicles larger than 5 mm diameter are practically non-existent in these ovaries.

An incomplete or abnormal cytoplasmic maturation could explain the high rates of polyspermic fertilization and low rates of embryo development observed in this study. Increases in polyspermic fertilization and parthenogenetic activation are 2 abnormalities indicating deficiencies in cytoplasmic maturation of oocytes obtained from young animals (17, 24). Damiani *et al*. (6) observed that calf oocytes exhibited some delay in organelle migration and redistribution following maturation. The most commonly observed anomaly involves different patterns of migration and dispersal of cortical granules, as reported by Damiani *et al*. (6) in calf oocytes and Wang *et al*. (38) in pig oocytes. On the other hand, Damiani *et al*. (6) suggested that abnormalities in calf oocytes could be due to an intrinsic deficiency. They also reported a limited Ca2+ release that could result in abnormal fertilization and decreased rates of development. These findings could help to explain the polyspermic fertilization reported in our experiment.

Fulka *et al*. (10) hypothesized that the acquisition of full developmental competence may extend beyond the growth phase and involves instead pre-maturation changes in the fully-grown oocytes. Based on our results, we suggest that whereas BCB is a useful test to select fully-grown oocytes that are able to mature to MII stage, most of them are unable to support normal embryonic development. We postulate that although BCB+ oocytes present higher percentages of normal fertilization compared to BCB- oocytes, their degree of cytoplasmic maturation is still low. Probably, these oocytes have not carried out completely the pre-maturation period to acquire a total developmental competence up to the blastocyst stage. Further studies are needed to develop an IVM system for prepubertal goat oocytes capable of achieving complete cytoplasmic maturation and provide the matured oocytes with the capability to support fertilization and early embryonic development.

It has been shown that interassay variability was reduced when immature oocytes were selected using the BCB test, indicating that application of the BCB test could enhance the sensitivity and maximize the reliability and efficiency of the homologous *in vitro* penetration assay (31). We suggest that the use of BCB selected oocytes for *in vitro* embryo production programs would allow the study of the specific needs for maturation of oocytes obtained from prepubertal animals. In conclusion, the BCB test is a useful method of selecting oocytes with larger size and, consequently, to select more competent immature prepubertal goat oocytes for *in vitro* embryo production programs.

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