Reprod. Nutr. Dev. 43 (2003) 179–187

DOI: 10.1051/rnd:2003012

Original article

Developmental competence of prepubertal goat oocytes selected with brilliant cresyl blue and matured with cysteamine supplementation

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(Received 2 January 2003; accepted 5 March 2003)

Abstract — The aim of this study was to assess the effect of oocyte selection using the brilliant cresyl blue (BCB) test plus the addition of cysteamine to the in vitro maturation (IVM) medium to improve the in vitro embryo development of prepubertal goat oocytes. The oocytes were exposed to 26 μM BCB and classified according to their cytoplasm coloration: BCB+ (oocytes with blue cytoplasm) and BCB– (unstained oocytes). The oocytes were matured in a conventional IVM medium supplemented with cysteamine 100 μM. The control group consisted of oocytes not exposed to BCB and matured without cysteamine. The IVM-oocytes were inseminated and cultured in synthetic oviductal fluid (SOF) for 7 days. The normal fertilisation rate (oocytes showing 2 pronuclei and 1 sperm tail) of BCB+ oocytes (40%) was higher than those of BCB– (21%) and control oocytes (22%). The percentage of morulae plus blastocysts was higher ($P < 0.05$) in the BCB+ group than in the BCB– group (23.8 vs. 5.1%, respectively). In conclusion, the integration of the BCB test and the addition of cysteamine in the protocol of in vitro embryo production from prepubertal goat oocytes has improved the developmental rates of embryo development.

embryo / IVF / IVM / thiol

1. INTRODUCTION

Brilliant Cresyl Blue (BCB) staining allows to determine the activity of glucose-6-phosphate dehydrogenase (G6PD) an enzyme synthesised in growing oocytes but with a decreased activity in oocytes that have finished their growth phase. Thus,

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oocytes that have finished their growth phase show a decreased G6PD activity and exhibit a cytoplasm with a blue coloration because they do not reduce BCB to a colourless compound. The BCB test has been used successfully to select oocytes for IVM-IVF in pigs [1, 2] and cows [3]. In our earlier study with prepubertal goat oocytes matured without cysteamine [4], we showed that oocytes exposed and stained with BCB are larger (136.6 vs. 125.5 μm in diameter) and more able to develop to morula and blastocysts than unstained oocytes (12.0 vs. 3.6%). However, blastocyst development rates were still lower (4% in BCB+ oocytes) than those obtained with oocytes from adult goats [5–9]. Prepubertal goat IVM-IVF-oocytes show a low incidence of male pronuclear (MPN) formation [10] and a high percentage of haploid embryos (59% of 2–4 cell embryos; [11]). The transformation of the fertilising sperm head into the MPN has been shown to be positively related to intracellular glutathione levels (GSH; [12]). De Matos et al. [13, 14], have shown that the addition of cysteamine to the maturation medium increases the GSH concentration in cow oocytes and improves the in vitro blastocyst yield. Moreover, the addition of cysteamine in the maturation medium increases the percentage of oocytes with decondensed sperm heads after IVF in pigs [15] and hamsters [16], and gives a higher percentage of embryo development in sheep [17] and pigs [15, 18]. Previous studies with prepubertal goat oocytes [19] have shown that 100 μM of cysteamine added to the maturation medium significantly improves the percentage of oocytes with 2 pronuclei (77.0% of total fertilised oocytes) compared to the control group (37.8%).

The aim of this study was to assess both effects together: oocyte selection using the BCB test plus the addition of cysteamine to the maturation medium to improve the in vitro embryo development of prepubertal goat oocytes.

2. MATERIALS AND METHODS

2.1. Oocyte collection

The ovaries were obtained from goats, 45 to 60 days old, from a local slaughterhouse and transported at 37 ºC in Dulbecco phosphate-buffered saline (PBS, P-4417, Sigma) containing 50 $\mu g \cdot mL^{-1}$ of gentamycin. The oocytes were recovered by slicing the ovaries in TCM199 (Sigma, B-2520), supplemented with 135 $\mu g \cdot mL^{-1}$ $NaHCO_3$, 11.1 $\mu g \cdot mL^{-1}$ heparin-sodium salt (H-3393, 170 $USP \cdot mg^{-1}$ Sigma), 2% (v/v) steer serum (Donor Bovine Serum®, CanSera, Ontario, Canada) and 50 $\mu g \cdot mL^{-1}$ gentamycin. Oocytes with one or more complete layers of cumulus cells and homogeneous cytoplasm were selected. Selected oocytes were randomly distributed among the treatment groups.

2.2. Brilliant cresyl blue test

Immediately after oocyte collection, the oocytes were washed 3 times in mPBS (PBS with 1090 $mg \cdot L^{-1}$ glucose, 35.2 $mg \cdot L^{-1}$ pyruvate, 0.4% (w/v) BSA, Sigma, A-9647, fraction V) and 50 $\mu g \cdot mL^{-1}$ gentamycin. Then the oocytes were exposed to 26 μM of BCB (Sigma, B-5388) diluted in mPBS for 90 min at 38.5 ºC in a 5% CO_2 humidified air atmosphere. Following BCB exposure, the oocytes were washed 3 times in mPBS and were classified into 2 groups, depending on their cytoplasm coloration; BCB+ oocytes showed a blue cytoplasm coloration and BCB– oocytes no blue coloration. After classification, the oocytes were washed 3 times in a maturation medium.

2.3. In vitro maturation of oocytes

The maturation medium was TCM199 (Sigma, M-7528) supplemented with 275 $\mu g \cdot mL^{-1}$ sodium pyruvate, 146 $\mu g \cdot mL^{-1}$ L-glutamine, 10% (v/v) steer serum, 10 $\mu g \cdot mL^{-1}$ o-LH (Sigma, L-5269), 10 $\mu g \cdot mL^{-1}$ o-FSH (Ovagen®, Immuno Chemicals Products

Ltd., Auckland, New Zealand), 1 μg·mL^{-1} 17β estradiol (Sigma, E-2257), 100 μM cysteamine (Sigma, M-9768) and 50 μg·mL^{-1} gentamycin. Groups of 20 to 25 oocytes were transferred to 100 μL microdrops of maturation medium and incubated for 27 h at 38.5 ºC in a humidified air atmosphere of 5% CO_2 under mineral oil (Sigma, M-3516). The control group consisted of oocytes not exposed to the BCB test and matured in the above maturation medium but without cysteamine.

2.4. Sperm preparation

At the end of the maturation period, the oocytes were inseminated with fresh semen. The ejaculates were collected from 2 Murciano bucks of proven fertility into artificial vaginas and were transported at 37 ºC within 30 min to the laboratory. The motility of the sperm cells was evaluated under an inverted microscope and the motile sperm fraction was separated by swim-up. Seventy microliters of semen was placed in each of several conical tubes under a 2 mL Defined Medium [20] modified by Younis et al. [21] and referred to as mDM here, and then incubated for 45 to 60 min in a humidified atmosphere of 5% CO_2 in air at 38.5 ºC. 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 × *g* for 10 min. After dis carding the supernatant, the resulting sperm pellet was resuspended 1:1 with mDM medium containing heparin (100 μg·mL^{-1} heparin-sodium salt; 170 USP·mg^{-1}) and was incubated for 45 to 60 min in a humidified air atmosphere of 5% CO_2 at 38.5 ºC (final concentration: 84 × 10^6 sperm·mL^{-1}, approximately).

2.5. In vitro fertilisation of the oocytes

After maturation, groups of 20 to 25 oocytes were transferred into 100 μL fertilisation microdrops of modified Tyrode medium (TALP), as described by Parrish et al. [22] supplemented with 1 μg·mL^{-1} hypotaurine (Sigma, H-1384) under mineral oil. After capacitation, the sperm concentration was assessed with a hemacytometer, and an aliquot (5 μL) of the sperm suspension was added to the fertilisation microdrops (final concentration: 3.5 × 10^6 sperm·mL^{-1}). We cultured it for 24 h in a humidified air atmosphere of 5% CO_2 at 38.5 ºC.

2.6. 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 maturation 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. The oocytes with a sperm in the cytoplasm were considered fertilised and were then classified into 1 of 3 groups: 2PN (female pronucleus, male pronucleus and sperm tail; normal fertilisation), polyspermy (2 or more sperm tails in the cytoplasm with condensed heads or two or more decondensed heads in the cytoplasm), and asynchrony (female pronucleus and a condensed sperm head).

The sperm heads were classified into 4 types according to their morphological changes in the cytoplasm [23]: Condensed head (Ch); Swollen head (Sh); Metaphase chromosomes of the sperm nucleus (Mc) and Male Pronucleus (MPN).

2.7. In vitro embryo culture

Following 24 h of sperm-exposure, the oocytes in the culture medium were washed with the aid of a fine pipette to separate the oocytes from any sperm cells. The embryos were cultured in groups of 20 to 25 embryos

in 20 to 25 µL microdrops (1 µL culture medium/embryo) of Synthetic Oviductal Fluid (SOF [24]) modified by Takahashi and First [25] in 35 mm culture dishes under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The 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, the total cell number of embryos was assessed with fluorescent microscopy after Hoechst staining and the percentage of cleaved embryos and the percentages of morulae and blastocysts were recorded.

2.8. Statistical analysis

The difference between the treatment groups was detected by Chi-square analysis or the Fischer exact test where appropriate. The overall chi-square was calculated and found to be significant before performing the Fischer exact test to detect differences between the treatment groups. The Kruskal-Wallis test was performed with the Dunn Multiple Comparison post-test to analyse the differences among groups in the embryo cell number, using the GraphPad InStat (version 3.01 for Windows 95, GraphPad Software, San Diego, California, USA). The differences with a probability value of 0.05 or less were considered significant.

3. RESULTS

The percentage of oocytes stained by BCB was 28.6% (360/1257). Table I shows the oocyte nuclear maturation after 27 h of IVM. The oocytes at the MII stage were 89.5% and 72.1% in BCB+ and BCB– oocytes, respectively. The total MII-oocytes matured with cysteamine (77.8%, $P < 0.05$) were higher than the control group (67.3%)

Table II shows the IVF parameters. Oocytes normally fertilised (2 PN) were higher in BCB+ than in BCB– and control group oocytes (39.7, 21.0 and 22.2%, respectively; $P < 0.001$). The oocytes with one condensed sperm head in their cytoplasm (asynchronous fertilisation) were higher in the control group than in the oocytes matured with cysteamine and exposed to BCB (13.9 vs. 6.5%, respectively; $P < 0.01$). We did not find differences among groups in polyspermic oocytes, with a number of 2.8, 2.7 and 2.8 spermatozoa/oocyte in BCB+, BCB– and the control group, respectively.

Tables III and IV show the morphological changes of the sperm head in the cytoplasm. In monospermic oocytes, the sperm heads that developed up to the

Table I. Effect of the BCB test and the addition of cysteamine in the IVM medium on the nuclear stage of in vitro matured oocytes (replicates = 3)[a].

Treatment	Total oocytes	GV *n* (%)	GVBD *n* (%)	MI *n* (%)	An-Tel *n* (%)	MII *n* (%)
Cysteamine						
BCB+	105	0 (0)	0 (0)	11 (10.5)b	0 (0)	94 (89.5)a
BCB–	219	3 (1.4)	1 (0.5)	57 (26.0)a	0 (0)	158 (72.1)bc
Total	324	3 (0.9)	1 (0.3)	68 (21.0)a	0 (0)	252 (77.8)b
Control	150	3 (2.0)	8 (5.3)	30 (20.0)ab	0 (0)	101 (67.3)c

[a] Values in the same column with different letters (a, b, c) differ significantly ($P < 0.05$). GV: Germinal vesicle; GVBD: Germinal vesicle breakdown; MI: Metaphase I; An-Tel: Anaphase-Telophase I; MII: Metaphase II.

Table II. Effect of the BCB test and the addition of cysteamine in the IVM medium on the in vitro fertilisation of in vitro matured oocytes (replicates = 3)[a].

Treatment	Inseminated oocytes	Fertilised oocytes, *n* (%)			
		Total	2 PN	Asynchronous	Polyspermic
Cysteamine					
BCB+	116	66 (56.9)	46 (39.7)a	2 (1.7)c	18 (15.5)
BCB–	286	132 (46.2)	60 (21.0)b	24 (8.4)ab	48 (16.8)
Total	402	198 (49.3)	106 (26.4)b	26 (6.5)bc	66 (16.4)
Control	144	78 (54.2)	32 (22.2)b	20 (13.9)a	26 (18.1)

[a] Values in the same column with different letters (a, b, c) differ significantly ($P < 0.05$). 2PN: two pronuclei + one sperm tail. Polyspermic: oocytes with two or more sperm tails in the cytoplasm with non-decondensed heads or two or more decondensed heads in the cytoplasm. Asynchronous: oocytes with a condensed sperm head and one feminine pronucleus.

Table III. Effect of the BCB test and the addition of cysteamine in the IVM medium on the morphological changes of sperm heads in monospermic oocytes (replicates = 6)[a].

Treatment	Monospermic oocytes, *n* (%)				
Cysteamine	Total oocytes	Male pronucleus	Metaphase chromosomes	Swollen head	Condensed head
BCB+	48	46 (95.8)a	0 (0)	0 (0)	2 (4.2)c
BCB–	84	60 (71.4)bc	0 (0)	6 (7.1)	18 (21.4)ab
Total	132	106 (80.3)b	0 (0)	6 (4.5)	20 (15.2)bc
Control	52	32 (61.5)c	0 (0)	0 (0)	20 (38.5)a

[a] Values in the same column with different letters (a, b, c) differ significantly (χ^2, $P < 0.05$).

Table IV. Effect of the BCB test and the addition of cysteamine in the IVM medium on the morphological changes of sperm heads in polyspermic oocytes (replicates = 6)[a].

Treatment	Sperm heads penetrating oocytes, *n* (%)				
Cysteamine	Total sperm heads	Male pronucleus	Metaphase chromosomes	Swollen heads	Condensed heads
BCB+	50	38 (76.0)	4 (8.0)a	2 (4.0)	6 (12.0)
BCB–	128	94 (73.4)	10 (7.8)a	8 (6.3)	16 (12.5)
Total	178	132 (74.2)	14 (7.9)a	10 (5.6)	22 (12.4)
Control	74	58 (78.4)	0 (0)b	2 (2.7)	14 (18.9)

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

male pronucleus were 95.8% in BCB+ and 71.4% in BCB– oocytes ($P < 0.01$). The addition of cysteamine to the maturation medium increased the formation of the male pronucleus (80.3%) compared to the control group (61.5%). In polyspermic oocytes (Tab. IV) we did not find these differences among the groups.

Table V. Effect of the BCB test and the addition of cysteamine in the IVM medium on the embryo development of in vitro matured and fertilised prepubertal goat oocytes (replicates = 6)[a].

Treatment	Inseminated oocytes	Embryo development at day 8 post-insemination				
Cysteamine		Cleaved embryos	≥ 8 cell embryos	Morulae + blastocyst	Blastocysts	
		n (%)	*n* (%)[b]	*n* (%)[b]	*n* (%)[b]	No. of cells
BCB+	139	63 (45.3)a	43 (68.3)a	15 (23.8)a	5 (7.9)	78.0 ± 14.4a
BCB–	392	137(34.9)b	29 (21.2)c	7 (5.1)b	4 (2.9)	43.3 ± 14.9b
Total	531	200 (37.6)ab	72 (36.0)b	22 (11.0)b	9 (4.5)	62.6 ± 22.8ab
Control	111	47 (42.3)ab	15 (31.9)bc	6 (12.8)ab	0 (0)	

[a]Values in the same column with different letters (a, b, c) differ significantly ($P < 0.05$). [b] Percentages calculated from cleaved embryos.

Table V shows the percentages of cleavage and development to the blastocyst stage of the oocytes.

With cysteamine in the maturation medium, BCB+ oocytes showed higher percentages of cleaved embryos (45.3%), embryos with eight or more cells (68.3%) and morula plus blastocysts (23.8%) than the BCB– oocytes (34.9%, $P < 0.05$; 21.1%, $P < 0.0001$ and 5.1%, $P < 0.001$, respectively). Cysteamine increased the blastocyst rate from 0 to 4.5%. The control group was not statistically different from the BCB+ oocytes in the morula plus blastocyst percentage but we did not find any blastocysts in the control group. The number of cells was higher in the blastocysts from the BCB+ than from the BCB– oocytes (78.0 ± 14.4 vs. 43.3 ± 14.9, $P < 0.05$, respectively).

4. DISCUSSION

This study shows that oocytes selected by the BCB test (BCB+ oocytes) and matured with cysteamine significantly improved the number of oocytes reaching the MII stage, oocytes with 2 PN, oocytes with MPN formation and embryos developing up to 8 cells, compared to the oocytes in the control group. Comparing oocytes exposed to the BCB test, BCB+ oocytes showed higher percentages of oocytes reaching MII, oocytes with 2 PN, oocytes with MPN formation, cleaved embryos, embryos developing beyond the 8-cell stage and the number of blastocysts cells than the BCB– oocytes. Previous studies in our laboratory [4] have shown that prepubertal goat oocytes stained with BCB show significantly higher rates of nuclear maturation and normal fertilisation (2PN oocytes) than unstained oocytes (BCB–), although the percentage of blastocysts is still low (4%). Pujol et al. [3] in calf oocytes have also shown increases in the percentages of the blastocyst yield of BCB+ compared to BCB– oocytes (12.3 vs. 1.6% respectively).

The total number of oocytes matured with cysteamine (BCB+ plus BCB–) showed higher percentages of nuclear maturation and MPN formation than oocytes matured without cysteamine (control group) but differences were not found in any of the other studied parameters. We suggest that cysteamine had a positive effect on embryo yield from prepubertal goats only when the BCB+ oocytes were used. Rodríguez-González et al. [4] observed that BCB+ oocytes were larger than BCB– oocytes. This could mean that complete oocyte intraovarian growth is needed before cysteamine

could have a positive effect on oocyte maturation.

Several studies have shown that the addition of cysteamine to the oocyte maturation medium increases intracytoplasmic GSH levels in different species [14, 17, 18, 26–28]. GSH, the major non-protein sulphydryl compound in mammalian cells, is an endogenous and ubiquitous reducing agent that protects cells from oxidation and plays a number of important roles in cellular metabolism including protein synthesis and the reduction of disulphides [29]. In oocytes, intracytoplasmic GSH participates in the transformation of the fertilising sperm head in the male pronucleus. It is believed that at fertilisation, a high intracellular GSH content is needed for the transformation of the disulphide-stabilised sperm nucleus into the male pronucleus [30, 31]. Indeed, the decrease in intracellular GSH of mouse oocytes impairs the decondensation of the sperm nucleus [32]. In prepubertal goat oocytes, the high incidence of asynchronous fertilisation (oocytes with a feminine pronucleus and intact sperm head) could be due to the low intracytoplasmic GSH levels of these oocytes. Previous studies with prepubertal goats have shown that the asynchronous fertilised oocytes develop to haploid embryos [11]. In our study the percentage of cleaved embryos in the control group (42.3%) was higher than the percentage of normally fertilised (2PN) oocytes (22.2%). With BCB+ oocytes, the difference between the percentage of cleaved embryos (45.3%) and the percentage of 2PN oocytes (39.7%) was reduced which suggests a lower number of haploid embryos obtained with BCB+ oocytes matured with cysteamine. Several authors have found an effect of intracytoplasmic GSH levels on MPN formation [15, 16, 28, 31, 33, 34]. In our study we observed significantly higher percentages of MPN in oocytes matured with cysteamine compared to the control group (80.3 vs. 61.5%, respectively).

In cattle [13, 14], buffalo [35] and sheep [17], the addition of cysteamine to the maturation media improves the in vitro blastocyst development. In pigs [15] the addition of 50 and 500 μM cysteamine increases the percentage of 2 PN oocytes (43 and 45% respectively vs. 10% in the control group) but the concentration of 500 μM also increases the percentage of blastocysts (12 vs. 1% in the control group). The explanation could be that both cysteamine concentrations affect the MPN formation but only the highest concentration further affects the mitotic development. Oocytes cannot incorporate cysteamine to synthesise GSH. Cysteamine acts by converting the cystine present in TCM199 (a cystine-rich medium) to cysteine and cysteine is incorporated by the oocyte to synthesise GSH (reviewed by Nagai [36]). Oocytes with or without a cumulus can take up cysteine. Mori et al. [37] have shown the effect of cumulus cells on intracytoplasmic GSH synthesis. Yamauchi and Nagai [18] have concluded that cysteamine increases the content of GSH and promotes MPN formation in cumulus-free porcine oocytes. Also, Grupen et al. [15] have reported a higher percentage of male pronucleus formation with a combination of TCM 199 and cysteamine. In our study, using 100 μM of cysteamine in TCM 199, the number of BCB+ oocytes that developed up to 8-cell embryos was higher than the control group. However, the differences were not statistically significant in morula and blastocyst yield. We suggest that in this study, the concentration of cysteamine causes an increase in GSH intracytoplasmic concentration to aid the sperm head decondensation but not enough to increase the blastocyst formation. In our previous study [4] using BCB+ selected oocytes matured in the same culture medium but without cysteamine, the percentage of oocytes reaching the morulae plus the blastocyst stage was 12% compared with the 23.8% reached in this study when adding cysteamine to the maturation medium.

In conclusion, the BCB test selects the oocytes that respond best to the addition of cysteamine to the IVM media. The selection of oocytes using the BCB test and the addition of cysteamine in the protocol of in vitro embryo production of prepubertal goat oocytes has improved the nuclear maturation, normal fertilisation (2PN), MPN formation and embryo development up to the morula stage.

ACKNOWLEDGEMENTS

This study was supported by CICYT (Grants AGF99-1232 and AGL2000-0353). The author E. Rodríguez-González was supported by a fellowship from the Generalitat de Catalunya.

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