Zygote **11** (November), pp. 347–354. © 2003 Cambridge University Press
DOI:10.1017/S0967199403002405 Printed in the United Kingdom

Supplementation with cysteamine during maturation and embryo culture on embryo development of prepubertal goat oocytes selected by the brilliant cresyl blue test

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Date submitted: 19.06.03. Date accepted: 12.09.03

Summary

Our previous studies have shown that the addition of 100 μM cysteamine to the *in vitro* maturation (IVM) medium increased the embryo development of prepubertal goat oocytes. The aim of the present study was to evaluate the effect of adding different concentrations of cysteamine to the IVM medium and to the *in vitro* embryo culture medium (IVC) on the embryo development of prepubertal goat oocytes selected by the brilliant cresyl blue (BCB) test. Oocytes were exposed to BCB and classified as: oocytes with a blue cytoplasm or grown oocytes (BCB+) or oocytes without blue cytoplasm or growing oocytes (BCB–). In Experiment 1, oocytes were matured in a conventional IVM medium supplemented with 100 μM, 200 μM or 400 μM cysteamine. In Experiment 2, oocytes were matured with 400 μM cysteamine and following *in vitro* fertilization (IVF) were cultured in SOF medium supplemented with 50 μM and 100 μM cysteamine. In Experiment 1, BCB+ oocytes matured with 100 μM and 200 μM cysteamine showed higher normal fertilization and embryo development rates than BCB– oocytes. Oocytes matured with 400 μM cysteamine did not present these differences between BCB+ and BCB– oocytes. In Experiment 2, the addition of 50 μM and 100 μM cysteamine to culture medium did not affect the proportion of total embryos obtained from BCB+ oocytes (35.89% and 38.29%, respectively) but was significantly different in BCB– oocytes (34.23% and 29.04%, respectively, $P < 0.05$). In conclusion, the addition of 400 μM cysteamine to the IVM improved normal fertilization and embryo development of BCB– oocytes at the same rates as those obtained from BCB+ oocytes. The proportions of morulae plus blastocyst development were not affected by the treatments.

Keywords: Goat, Cysteamine, Prepubertal, IVF, IVC

Introduction

Cysteamine is a low molecular weight thiol that, when present during *in vitro* maturation (IVM) and *in vitro* culture (IVC) of embryos, increases the intracytoplasmic oocyte glutathione (GSH) concentration and improves embryo development rates (De Matos *et al*., 1995; Luvoni *et al*., 1996; De Matos and Furnus, 2000). GSH participates in various mechanisms such as amino acid transport, protein synthesis, reduction of disulphides and protection against oxidative damage. Addition of cysteamine to the IVM medium of cow oocytes improves *in vitro* blastocyst development (De Matos *et al*., 1995, 1996). Similar results were found in sheep (De Matos *et al*., 2002*b*), buffalo (Gasparrini *et al*., 2000), pig (Grupen *et al*., 1995; Yamauchi and Nagai, 1999) and mouse (De Matos *et al*., 2003). Previous studies in our laboratory (Rodríguez-González *et al*., 2003*a*) showed that 100 μM cysteamine added to maturation medium significantly improved the proportion of male pronucleus (MPN) formation in *in vitro* fertilization (IVF) oocytes of prepubertal goats, but embryo development was not increased. In cows, cysteamine supplementation of the IVC medium increased the proportion of blastocyst development by stimulating intracellular glutathione synthesis (De Matos *et al*., 2002*a*).

Oocytes recovered from ovaries of slaughtered prepubertal goats are heterogeneous. Brilliant cresyl blue (BCB) stain permits us to determine the activity

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of glucose-6-phosphate dehydrogenase (G6PD), an enzyme synthesized in growing oocytes but with decreased activity in oocytes that have finished their growth phase. Thus, oocytes that have finished their growth phase show 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 (Ericsson *et al*., 1993; Roca *et al*., 1998) and heifers (Pujol *et al*., 2000). In our earlier studies with prepubertal goat oocytes (Rodríguez-González *et al*., 2002), we showed that oocytes exposed and stained with BCB were larger (136.6 μm vs 125.5 μm diameter) and increased the proportion of oocytes with MPN compared with unstained oocytes. Also, we have demonstrated that oocytes supplemented with 100 μM cysteamine increased the proportion of BCB+ oocytes that developed further to 8-cell embryos (Rodríguez-González *et al*., 2003*b*). However, blastocyst development rates were still lower in BCB+ oocytes than those obtained with oocytes from adult goats (Cognié *et al*., 1995; Crozet *et al*., 1995; Pawshe *et al*., 1996; Kenkistepe *et al*., 1996, 1998). The effect of cysteamine on *in vitro* embryo production has different effects depending of the concentration, the species and the type of oocyte used in study (Grupen *et al*., 1995; De Matos *et al*., 2002*b*, 2003).

With the aim of trying to improve *in vitro* embryo production (IVEP) from prepubertal goat oocytes, two experiments were designed – Experiment 1, with the aim of evaluating the effects on embryo development of BCB-selected oocytes of using different concentrations of cysteamine in the IVM medium (100 μM, 200 μM and 400 μM), and Experiment 2, with the aim of evaluating the effects on embryo development of BCB-selected oocytes of using different concentrations of cysteamine in the IVC medium (50 μM and 100 μM).

Materials and methods

Oocyte collection

Ovaries were obtained from 45–60-day-old goats from a local slaughterhouse and transported at 37 °C in Dulbecco's PBS (P-4417, Sigma) containing 50 μg ml^{-1} of gentamycin. Oocytes were recovered by slicing the ovaries in TCM199 (M-2520, Sigma), supplemented with 2.2 mg ml^{-1} $NaHCO_3$, 2% (v/v) steer serum (Donor Bovine Serum®, CanSera, Ontario, Canada) and 50 μg ml^{-1} gentamycin. Oocytes with one or more complete layers of cumulus cells and homogeneous cytoplasm were selected. Selected oocytes were randomly distributed among treatment groups.

BCB test

Immediately after oocyte collection, oocytes were washed three times in mPBS [PBS with 1090 mg l^{-1} glucose, 35.2 mg l^{-1} sodium pyruvate, 0.4% (w/v) bovine serum albumin (A-9647, fraction V, Sigma) and 50 μg ml^{-1} gentamycin]. Then, the oocytes were exposed to 26 μM BCB (B-5388, Sigma) diluted in mPBS for 90 min at 38.5 °C in humidified air containing 5% CO_2. Following BCB exposure, oocytes were washed three times in mPBS and classified into two groups, depending on their cytoplasm coloration: BCB+ oocytes with blue cytoplasm and BCB– oocytes without blue coloration. After classification, oocytes were washed three times in maturation medium.

IVM of oocytes

The maturation medium was TCM199 (M-7528, Sigma) supplemented with 275 μg ml^{-1} sodium pyruvate, 146 μg ml^{-1} L-glutamine (G-5763, Sigma), 10% (v/v) steer serum, 10 μg ml^{-1} ovine-LH (L-5269, Sigma), 10 μg ml^{-1} ovine-FSH (Ovagen®, Immuno Chemicals Products, Auckland, New Zealand), 1 μg ml^{-1} 17β Hestradiol (E-2257, Sigma), 100 μM, 200 μM or 400 μM cysteamine (M-9768, Sigma) and 50 μg ml^{-1} gentamycin. Groups of 20–25 oocytes were transferred to 100 μl microdrops of maturation medium and incubated for 25 h at 38.5 °C in humidified air with 5% CO_2 under mineral oil (M-8410, Sigma).

Sperm preparation

At the end of the maturation period, oocytes were inseminated with fresh semen. Ejaculates were collected from two Murciano bucks of proven fertility into artificial vaginas and transported at 37 °C to the laboratory within 30 min. The motility of sperm cells was evaluated under an inverted microscope and the motile sperm fraction separated by swim-up. 70 μl of semen was placed in each of several conical tubes under 2 ml Defined Medium (Brackett and Oliphant, 1975) as modified by Younis *et al*. (1991), here referred to as mDM. Spermatozoa were incubated for 45–60 min in humidified air with 5% CO_2 at 38.5 °C. 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 discarding the supernatant, the resulting sperm pellet was resuspended 1:1 with mDM medium containing heparin (100 μg ml^{-1} heparin sodium salt) and incubated it for 45–60 min in humidified air with 5% CO_2 at 38.5 °C (final concentration approximately 84×10^6 sperm ml^{-1}).

IVF 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*. (1986), supplemented with 1 μg ml^{-1}

https://doi.org/10.1017/S0967199403002405 Published online by Cambridge University Press

hypotaurine (H-1384, Sigma) under mineral oil. After capacitation, sperm concentration was assessed with a haemacytometer and a 5 μl aliquot of the sperm suspension was added to the fertilization microdrops (final concentration: 3.5×10^6 sperm ml^{-1}). Gametes were cultured for 24 h in humidified air with 5% CO_2 at 38.5 °C.

Evaluation of oocytes after IVM and IVF

To evaluate the nuclear stage after maturation a sample of oocytes was fixed at 25 h of IVM and stained it with 1% lacmoid (L-7512, Sigma). Oocyte maturation was measured by the proportion of oocytes reaching the metaphase II (MII) stage.

To evaluate the pronuclear stage after 17 h of IVF, a sample of oocytes was processed in the same way as the oocytes fixed after IVM. Oocytes with a sperm in the cytoplasm were considered to be fertilized and classified into three groups: 2PN (female pronucleus, male pronucleus and one sperm tail; normal fertilization), polyspermy (two 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).

In vitro embryo culture

Following 24 h of sperm exposure, oocytes were washed and denuded with the aid of a fine pipette to separate oocytes from any sperm cells. Embryos were cultured in groups of 20–25 embryos in 20–25 μl microdrops (1 μl culture medium per embryo) of synthetic oviductal fluid (SOF; Tervit *et al*., 1972) as modified by Takahashi & First (1992) in 35-mm culture dishes under mineral oil in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2. Presumptive zygotes were maintained in culture for 7 days (Experiment 1). 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 per embryo). In Experiment 2, the presumptive zygotes were cultured for 24 h (day 1) in SOF. At the end of day 1, embryos were washed twice in SOF and transferred to SOF with 50 μM or 100 μM cysteamine and cultured for 48 h (days 2–3). At the end of day 3, the medium was replaced and embryos were cultured with SOF without cysteamine for another 5 days in the culture medium.

At the end of the culture period, the total cell number of embryos was assessed with fluorescence microscopy after Hoechst staining and the percentage of embryos at different developmental stages was recorded.

Statistical analysis

The difference between treatment groups was detected by χ^2 analysis or Fisher's exact test, where appropriate – the overall χ^2 score was calculated and found to be significant before performing the Fisher's exact test to detect differences between treatment groups, using GraphPad InStat [version 3.01 for Windows95, GraphPad Software (San Diego, California, USA)]. Differences with a probability of 0.05 or less were considered to be significant.

Experimental design

Experiment 1

We analysed the effect on embryo development of 100 μM, 200 μM and 400 μM cysteamine added to the IVM medium of prepubertal goat oocytes. Experiment 2 was designed based on the results obtained in Experiment 1.

Experiment 2

We analysed the effect of adding 50 μM or 100 μM cysteamine for 48 h (days 2–3 of IVC) on embryo development of oocytes from prepubertal goats matured with 400 mM cysteamine.

Results

Experiment 1

The proportion of oocytes stained by the BCB test (BCB+ oocytes) was 48.06% (1140/2372). Table 1 shows the results of nuclear stage IVM oocytes and IVF oocytes. The proportion of oocytes at MII stage was higher in BCB+ oocytes than BCB– oocytes, in each one of the cysteamine concentrations studied. No significant differences in the proportion of total fertilized oocytes were detected among cysteamine groups and BCB-selected oocytes. The proportion of 2PN oocytes was higher ($P < 0.05$) in BCB+ oocytes than BCB– oocytes in the 100 μM and 200 μM cysteamine groups (86.4% vs 58.8% and 86% vs 65.6%, respectively). Oocytes matured with 400 μM cysteamine did not show differences in the proportion of 2PN oocytes between BCB+ and BCB– oocytes (78.78% vs 71.42%, respectively).

Table 2 shows the proportions of embryo development of prepubertal goat oocytes. BCB+ oocytes matured with 100 μM and 200 μM cysteamine showed higher proportions ($P < 0.05$) of total embryos (36.5% and 33.9%, respectively) than BCB– oocytes (17.9% and 11%, respectively). However, oocytes matured with 400 μM cysteamine did not show these differences between BCB+ and BCB– oocytes (35.7% and 29.3%, respectively). At the end of day 8 of IVC, the highest

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Table 1 Effect of oocyte selection using the brilliant cresyl blue (BCB) test and different concentrations of cysteamine added to the IVM medium on nuclear stage of IVM and IVF oocytes of prepubertal goats (nine replicates). Columns show numbers, with percentages in brackets

	IVM		IVF				
Treatment	Total oocytes	Metaphase II	Total oocytes	Total	2PN*	Asynchronous*	Polyspermic*
100 μM							
BCB+	131	89 (67.93%)^{ae}	101	37 (36.63%)	32 (86.48%)^{a}	2 (5.40%)	3 (8.10%)
BCB−	128	74 (57.81%)^{bd}	101	34 (33.66%)	20 (58.82%)^{b}	6 (17.64%)	8 (23.52%)
200 μM							
BCB+	153	134 (87.58%)^{c}	102	43 (42.15%)	37 (86.04%)^{a}	2 (4.65%)	4 (9.30%)
BCB−	152	108 (71.05%)^{a}	105	32 (30.47%)	21 (65.62%)^{b}	2 (6.25%)	9 (28.12%)
400 μM							
BCB+	144	111 (77.08%)^{a}	100	33 (33.0%)	26 (78.78%)^{ab}	2 (6.06%)	5 (15.15%)
BCB−	153	94 (61.43%)^{de}	104	35 (33.65%)	25 (71.42%)^{ab}	1 (2.85%)	9 (25.71%)

2PN, two pronuclei and one sperm tail; asynchronous, oocytes with a condensed sperm head and one female pronucleus; polyspermic, oocytes with two or more sperm tails in the cytoplasm with nondecondensed heads or two or more decondensed heads in the cytoplasm.

$^{a-e}$Values in the same column with different letters differ significantly ($P < 0.05$).

*Percentages calculated from total fertilized oocytes.

proportion of morulae plus blastocysts was obtained from BCB+ oocytes matured with 400 μM cysteamine, but this proportion was not statistically different of those obtained from BCB– oocytes.

Experiment 2

The proportion of oocytes stained by the BCB test was 42.0% (764/1819). Table 3 shows the proportion of embryo development of oocytes matured with 400 μM cysteamine and cultured in SOF medium supplemented with 50 μM and 100 μM cysteamine. No significant differences in total embryos were detected between BCB+ and BCB– oocytes cultured with 50 μM cysteamine (35.89% and 34.23%, respectively), but there were differences in the 100 μM cysteamine group (38.29% and 29.04%, respectively; $P < 0.05$). The proportion of morulae plus blastocysts obtained was similar among groups.

Discussion

This study demonstrated that BCB+ oocytes matured with 100 μM and 200 μM cysteamine significantly increased the proportion of oocytes reaching MII, the number of normal fertilized oocytes (2PN oocytes)

Table 2 Effect of oocyte selection using the brilliant cresyl blue (BCB) test and different concentrations of cysteamine added to the IVM medium on embryo development of prepubertal goat oocytes (nine replicates). Columns show numbers, with percentages in brackets

		Embryo development at day 8 after insemination			
Treatment	Inseminated oocytes	Total embryos	Two- to seven-cell embryos*	Eight- to 16-cell embryos*	Morulae + blastocysts*
100 μM					
BCB+	203	74 (36.45%)^{a}	56 (75.67%)	15 (20.27%)	3 (4.05%)^{cd}
BCB−	195	35 (17.94%)^{b}	30 (85.71%)	3 (8.57%)	2 (5.71%)^{ce}
200 μM					
BCB+	221	75 (33.93%)^{a}	64 (85.33%)	9 (12.0%)	2 (2.66%)^{cd}
BCB−	253	28 (11.06%)^{b}	25 (89.28%)	2 (7.14%)	1 (3.57%)^{cde}
400 μM					
BCB+	204	73 (35.78%)^{a}	51 (69.89%)	12 (16.43%)	10 (13.69%)^{e}
BCB−	215	63 (29.30%)^{a}	50 (79.36%)	10 (15.87%)	3 (4.76%)^{de}

$^{a-e}$Values in the same column with different letters differ significantly ($P < 0.05$).

*Percentages calculated from total embryos.

https://doi.org/10.1017/S0967199403002405 Published online by Cambridge University Press

Table 3 Effect of oocyte selection using the brilliant cresyl blue (BCB) test and different concentrations of cysteamine added to the IVC medium on embryo development of prepubertal goat oocytes (nine replicates). Columns show numbers, with percentages in brackets

Treatment	Inseminated oocytes	Embryo development at day 8 after insemination			
		Total embryos	Two- to seven-cell embryos*	Eight- to 16-cell embryos*	Morulae + blastocysts*
50 μM					
BCB+	273	98 (35.89%)[a]	79 (80.61%)	13 (13.26%)	6 (6.12%)
BCB−	368	126 (34.23%)[a]	105 (83.33%)	17 (13.49%)	4 (3.17%)
100 μM					
BCB+	282	108 (38.29%)[a]	92 (85.18%)	12 (11.11%)	4 (3.70%)
BCB−	396	115 (29.04%)[b]	101 (87.82%)	11 (9.56%)	3 (2.60%)

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

and total number of embryos obtained compared with BCB– oocytes. However, these differences between BCB+ and BCB– oocytes were not found in oocytes matured with 400 μM cysteamine. With prepubertal goat oocytes matured without cysteamine, we have already shown that BCB+ oocytes were larger and more competent for embryo development than BCB– oocytes. The presence of 100 μM cysteamine in the IVM medium significantly increased the number of fertilized oocytes with MPN formation (Rodríguez-González *et al*., 2003*a*) compared with matured oocytes without cysteamine. Based on our results, we conclude that high concentrations of cysteamine in the IVM medium (400 μM) increased embryo development of BCB– oocytes at the same rate as BCB+ oocytes. Consequently, more oocytes (BCB+ plus BCB–oocytes) for an *in vitro* embryo production program would be available from prepubertal goat ovaries.

In experiment 2, we show that the addition of 50 μM and 100 μM cysteamine to the IVC medium has no effect on embryo development. Comparing the results from both experiments (Experiments 1 and 2), we observe that the total number of embryos obtained from overall oocytes matured with 400 μM cysteamine in experiment 1 (32.4%, 136/419) was similar to those obtained in Experiment 2, in which embryos were cultured with 50 μM (34.9%, 224/641) or 100 μM (32.8%, 223/678) cysteamine.

In conclusion, the supplementation of the IVC medium with cysteamine during the second and third days after insemination did not affect embryo development of prepubertal goat oocytes matured with 400 μM cysteamine. In our previous study, the addition of glucose and GSH to IVC did not affect the embryo development of these oocytes (Urdaneta *et al*., 2003). Oocytes cannot incorporate cysteamine to synthesize GSH. Cysteamine acts by converting the cystine present in TCM199 (a cystine-rich medium) to cysteine, and cysteine is incorporated by the oocyte into GSH synthesis (Nagai, 2001). We have shown that the presence of cysteamine, cysteine, cystine and β-mercaptoethanol to the IVM medium of prepubertal goat oocytes increased intracellular GSH levels (Rodríguez-González *et al*., 2003*b*). Oocytes with or without cumulus can take up cysteine. Mori *et al*. (2000) showed the effect of the cumulus cells on intracytoplasmic GSH synthesis, and Yamauchi and Nagai (1999) concluded that cysteamine increased the content of GSH and promoted MPN formation in cumulus-free porcine oocytes. Several studies have shown that the addition of cysteamine to the oocyte maturation medium increases intracytoplasmic GSH levels in different species (De Matos *et al*., 1996, 1997, 2000, 2002*a*,*b*; Nagai *et al*., 1996; Yamauchi & Nagai, 1999; Rodríguez-González *et al*., 2003*b*). GSH, the major non-protein sulfhydryl compound in mammalian cells, is an endogenous, ubiquitous reducing agent that protects cells from oxidation (Lafleur *et al*., 1994). In prepubertal goat oocytes, the high incidence of asynchronous fertilization (oocytes with feminine pronucleus and intact sperm head) found in our previous studies (Mogas *et al*., 1997) could be due to the low intracytoplasmic GSH levels of these oocytes. The addition of cysteamine to the IVM medium has reduced this fertilization anomaly of prepubertal goat oocytes (Rodríguez-González *et al*., 2003*a*). Several authors have found an effect of intracytoplasmic GSH levels on MPN formation (Yoshida *et al*., 1993; Funahashi *et al*., 1994; Perreault *et al*., 1994; Grupen *et al*., 1995; Nagai *et al*., 1996; Kito & Bavister, 1997). In cattle (De Matos *et al*., 1995, 1996), buffalo (Gasparrini *et al*., 2000) and sheep (De Matos *et al*., 2002*b*), the addition of cysteamine to the maturation media improved *in vitro* blastocyst development. In pig (Grupen *et al*., 1995), the addition of 50 μM or 500 μM cysteamine increased the proportion of

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2PN oocytes (43% and 45%, respectively, vs 10% in the control group), but a 500 μM concentration also increased the proportion of blastocysts (12% vs 1% control group). This might be because both cysteamine concentrations affected the MPN formation but only the highest concentration affected further embryonic development. In our study, with 400 μM cysteamine added to IVM medium, the proportion of total embryos obtained from BCB– oocytes was significantly increased compared with BCB+ oocytes. The proportion of morulae plus blastocysts was not affected by cysteamine concentration or oocyte type. Cysteamine supplementation to the IVC medium did not improve blastocyst development. De Matos *et al*. (2003) tested different cysteamine concentrations added to the IVM medium and observed the positive effect of cysteamine on blastocyst development of oocytes from adult mice, but this effect was not observed in oocytes recovered from prepubertal females. Also, these authors reported the positive effect on blastocyst development of cysteamine addition to the IVC medium of adult mouse embryos. This effect was not observed in oocytes from prepubertal mice. However, in both experiments (cysteamine addition to IVM and IVC media), the blastocyst rates obtained from prepubertal mice oocytes matured and cultured without cysteamine were similar to those obtained from ovulated oocytes. The authors suggest that the oocyte population isolated from prepubertal mice was of a higher quality than oocytes recovered from adult mice. This high oocyte quality would not need cysteamine supplementation to develop up to blastocyst stage. The low number of morulae plus blastocysts found in our study with prepubertal goat oocytes could be due to a lower quality of oocytes obtained from prepubertal goat ovaries recovered from a commercial slaughterhouse. In goats, Wang *et al*. (2002) studied embryo development of IVM-IVF-IVC oocytes from gonadotropin-primed prepubertal and adult females, and observed significant differences in blastocyst development (6% vs 16%, respectively) but no differences in recipient pregnancy rates and proportion of kids born were observed following embryo transfer. Baldasarre *et al*. (2002, 2003) obtained transgenic kids born from microinjected IVM-IVF zygotes from gonadotropin-primed prepubertal goats. Recently, Koeman *et al*. (2003) observed significantly more oocytes recovered from gonadotropin-primed prepubertal goats than gonadotropin-primed adults, and no significant differences in blastocyst development assessed by morphological appearance (25% vs 24%, respectively). However, these proportions were reduced (6% and 7%, respectively) when assessing embryo development using cell counts.

In conclusion, prepubertal goat oocytes selected by the BCB test (BCB+ oocytes) and matured with 100 μM and 200 μM cysteamine supplementation significantly improved the proportion of normal fertilized oocytes and the proportion of total embryos obtained compared with BCB– oocytes. However, the addition of 400 μM cysteamine to the IVM medium allows an increase in the proportion of normal fertilized oocytes and the total number of embryos obtained from BCB– oocytes at the same proportions as those obtained from BCB+ oocytes. Consequently, this supplementation might increase the number of oocytes per prepubertal goat ovary used for *in vitro* embryo production. The addition of cysteamine to the IVC medium had no effect on the embryo development of prepubertal goat oocytes. Further studies are necessary to identify *in vitro* conditions able to support development of goat oocytes to the blastocyst stage.

Acknowledgements

This study was supported by MCyT (Grant AGL2000-0353).

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https://doi.org/10.1017/S0967199403002405 Published online by Cambridge University Press