I J Biotech., June (2003) 621-626

The effectivity of CR1aa medium on in vitro maturation, fertilization and early embryo development of goat oocyte

Yohan Rusiyantono[1] and Arief Boediono[2*]

1) Lab. of Reproduction, Dept. of Animal Husbandry, Fact. of Agriculture, Tadulako University, Bumi Tadulako Tondo Campus, Palu, Indonesia.

2) Lab. of Embryology, Dept. of Anatomy, Fact. of Veterinary Medicine, Bogor Agricutural University, IPB Darmaga Campus, Bogor, Indonesia. E-mail: ab1@cbn.net.id

Abstract

The aim of the present study was to compare different media for supporting *in vitro* maturation, fertilization and early embryo development of goat oocyte. Two kinds of media, namely TCM 199 and CR1aa media were used for *in vitro* maturation and embryos culture. Three different media, BO, TALP and CR1aa were used for *in vitro* fertilization. Oocytes were matured in TCM 199 or CR1aa media, respectively, for 24 hours at 38.5°C in 5% CO_2 incubator. *In vitro* fertilization was done in 5% CO_2 incubator at 38.5°C using fresh ejaculated sperm. After 8 hours of insemination, zygotes were cultured in two kinds of culture media, namely TCM-199 and CR1aa media, respectively in 5% CO_2 incubator up to day-5. The results showed that no significant difference in the percentages of oocytes reaching metaphase II in the two maturation media. The fertilization rate in CR1aa medium (63.2%) was significantly higher ($P<0.05$) than BO and TALP medium (48.9% and 50.0%, respectively). The cleavage rate and embryos development in CR1aa medium were 49.76% and 39.63%, they were significant ($P<0.05$) than those in TCM 199 medium (40.84% and 29.58%).

Keywords: CR1aa – goat – maturation – fertilization – development.

Introduction

Efficient *in vitro* procedures for oocytes maturation and fertilization in large domestic species are important for development of new biotechnological protocol such as gene transfer and *in vitro* multiplication of identical embryos (De Smedt *et al.*, 1992). Techniques for production of embryos are being widely used in numerous laboratories. Although the *in vitro* maturation and fertilization oocytes for two other domestic ruminant species (cattle and sheep) have been extensively investigated (Crozet *et al.*, 1987; Boediono *et al.*, 1944), information on the production of goat embryos from *in vitro* matured and fertilized oocytes is limited.

*) Corresponding author: Arief Boediono. Address: Lab. of Embryology, Dept. of Anatomy, Fact. of Veterinary Medicine, Bogor Agricutural University, Jl. Agatis, IPB Darmaga Campus, Bogor 16680, Indonesia. Telephone : (0251) 421-823; Facsimile: (0251) 421-823; E-mail : ab1@cbn.net.id

The culture medium employed in IVM not only affects the proportion of oocytes that reach metaphase II and become capable of undergoing fertilization, but also influences subsequent embryonic development (Bavister *et al.*, 1992). From the previous results of Izquierdo *et al.* (1998), the percentages of goat oocytes matured in medium TCM supplemented with serum and hormones was 66.34%, while the fertilization and cleavage rate obtained were 20.00% and 22.6%, respectively. The media employed for IVF must be capable of providing sperm motility and capacitation, the union of gametes, and the start of embryo development.

The objective of the present study was to examine the effectivity of CR1aa medium for *in vitro* maturation, fertilization and early embryo development of goat oocyte.

Materials and Methods

Oocyte Collection and Maturation

Goat ovaries were collected from a local slaughterhouse and transported at 30-35°C in NaCl physiology, containing Penicillin-G (1000 IU/µl) and Streptomycin Sulfate (0.2µg/l). Cumulus-oocytes complexes (COCs) were aspirated from antral follicles of 2 to 5 mm in diameter using 5-ml disposable plastic syringe fitted with an 18-G needle, and then transferred to a petri dish containing modified-Phosphate Buffer Saline (m-PBS, Gibco, USA) supplemented with 0.3% Bovine Serum Albumin (BSA, Sigma USA), and 50 mg/l gentamicin (Gibco, USA). The aspirated COCs were washed three times and incubated in two maturation media. Twenty oocytes were placed in 100-µl micro drops of the maturation medium, covered with sterile mineral oil and incubated at 38.5°C under 5% CO_2 in air.

In vitro Fertilization

Matured oocytes were fertilized *in vitro* by using fresh sperm in three different fertilization media. Spermatozoa were washed twice by centrifugation (500 x g, 5 min) in fertilization media without BSA supplementation. The sperm pellet was resuspended in fertilization media supplemented with 6 mg BSA/ml, 20 µg/ml heparin (Sigma, USA) and 2.5 mM caffeine (Sigma, USA). The spermatozoa at a final concentration of 5×10^6 spermatozoa/ml were added to each 50 µl fertilization droplet. Oocytes matured *in vitro* were transferred into sperm microdroplets (10 to 20 oocytes per microdroplet). Fertilization was done by incubation the sperm-oocytes for 18 hours at 38.5°C under 5% CO_2 in air.

Embryo Culture

After 18 hours of insemination, oocytes were washed with culture medium and transferred into 50 µl droplets of culture medium for further development. Adherent cumulus cells surrounding the embryos were removed by repeated pipetting 48 h post-fertilization. The cumulus cells adhering to the surface of the culture dish were not disrupted, and embryos were cultured on this somatic cell monolayer. The culture medium was replaced with fresh medium 96 h post-fertilization.

Experimental Design

Experiment I. A total of 370 oocytes were incubated *in vitro* during 18, 22, 26 and 30 hours in two different maturation media namely: a) Tissue Culture Medium-199 (TCM, Gibco, USA) or b). C. Rosenkrans 1 amino acid medium (CR1aa) (Rosenkrans and First, 1994). Maturation media were supplemented with 0.01 mg/ml follicle stimulating hormone (FSH, Denka Pharma-

ceutical, Japan), 10% (v/v) inactivated new born-calf serum (NCS, Sigma, USA) and 50 µg/ml gentamicin sulfate. The capability of goat oocytes to mature (reach the metaphase II) was evaluated by aceto-orcein staining to determine the nuclear development (Boediono *et al.*, 2003). After incubation for maturation, the oocytes were fixed in acetic acid-etahanol (1:3) for 3 days, stained with 1% aceto-orcein and cleared wuth aceto-glycerol. The oocytes were examined under a phase contrast microscope to determine the percentage of oocytes that developed to Germinal Vesicle (GV), Germinal Vesicle Break Down (GVBD), Metaphase-I (M-I) and Metaphase-II (M-II) stages.

Experiment II. A total of 266 matured oocytes were fertilized by using spermatozoa collected from fresh ejaculated goat in three different fertilization media namely: a) BO (Brackett and Oliphant, 1975), b) Modified TALP (Parish *et al.*, 1986), or c) CR1aa. Fertilization rate of matured oocytes was evaluated by aceto-orcein staining to determine the pronuclear status. Pronuclear status was evaluated 18 hours after fertilization by aceto-orcein staining, using the same method as described above. The fertilization rate was calculated as the percentage of stained oocytes that had two pronuclei (normal fertilization) and oocytes that had more than two pronuclei (polyploidy).

Experiment III. A total of 770 zygotes were further cultured in two different media namely: 1) Medium TCM-199 supplemented with 10% NBS, 5 µg/ml insulin (Wako Pure Chemical Industries, Osaka, Japan), and gentamicin 50 µg/ml) or 2) CR1aa supplemented 0.3 % BSA, 5 µg/ml insulin and gentamicin 50 µg/ml. The cleavage rate was evaluated 48 hours post-fertilization and development of cleaved embryos up to morula was observed on day 5 and 6, respectively.

Statistical Analyses

Each treatment was repeated 3 times, and data from experiment I, II and III were analysis by Chi-Square analysis. In this study, mean values were considered to be significantly different when P was set at the $P<0.05$ level.

Results and Discussion

The maturation rate (M-II) of collected goat oocytes incubated in the two maturation media were not significantly different ($P>0.05$), i.e. 78.3% and 76,5% in TCM-199 and CR1aa medium, respectively (Table 1). Supplementation of serum and gonadothropins such as FSH, usually cause synergistic enhancement of nuclear maturation, depending on the type of serum supplement used in the maturation medium (Pawshe *et al.*, 1996).

Table 1. Nuclear status of goat oocytes after maturation in different maturation media

Incubation periods	Maturation media	No. of Oocytes	Nuclear Status (%) GV	GVBD	M-I	M-II	PN	UI
18 h	TCM199	45	2(4.4)	9(20.0)	12(26.7)	20(44.5)[a]	-	2(4.4)
	CR1aa	40	3(7.5)	7(17.5)	11(27.5)	17(42.5)[a]	-	2(5.0)
22 h	TCM199	50	-	4(8.0)	7(14.0)	36(72.0)[a]	-	3(6.0)
	CR1aa	50	2(4.0)	3(6.0)	8(16.0)	34(68.0)[a]	-	3(6.0)
26 h	TCM199	45	-	1(2.2)	5(11.1)	37(82.3)[a]	-	2(4.4)
	CR1aa	50	-	-	5(10.0)	44(88.0)[a]	-	1(2.0)
30 h	TCM199	45	-	-	6(13.4)	36(80.0)[a]	1(2.2)	2(4.4)
	CR1aa	45	-	-	5(11.1)	38(84.5)[a]	2(4.4)	-

Note: GV= Germinal Vesicle; GVBD=Germinal Vesicle Break Down; M-I=Metaphase-I; M-II= Metaphase-II; PN=Pronucleus; UI = Un-identified. Values within columns with the same superscripts are no significantly difference ($P>0.05$)

The success of oocytes maturation is mainly indicated by the condition of the medium in which the oocyte is able to reach maturity level (Figure 1A). The presence of pyruvate, glutamine and glucose in the maturation medium can increase energy availability employed in the oxidative

metabolism process within mitochondria. Oxidative metabolism within mitochondria is the main energy resource in the process of oocyte maturation (Reiger and Lokskutoff, 1994). The maturation medium also has an important role in the continuation of IVF process. Culture medium employed in the oocyte maturation not only influences the number of oocyte which can achieve M-II phase, but can also increase fertilization capability and future embryonic development (Bavister *et al.*, 1992; Totey *et al.*, 1993).

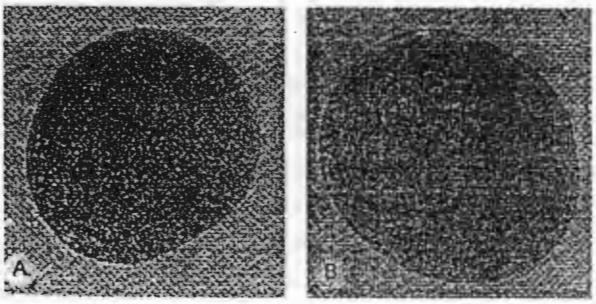

Figure 1. Nuclear status of goat oocytes. A. Matured oocyte with the presence of chromosome (CH) and first polar body (PB), 22 hours after of *in vitro* maturation. B. Fertilized oocyte with the presence of two pronuclei (PN), 18 hours after *in vitro* insemination.

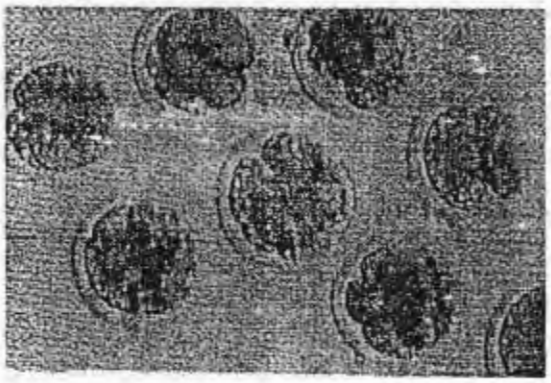

Figure 2. Development of *in vitro* maturation, fertilization and culture goat oocytes on Day-2. Embryos developed to cleavage (2-, 4- and 8-cell stages).

The success of IVF program is highly determined by the oocyte maturation and sperm quality used in fertilization. The

Table 2. Fertilization rate of goat oocytes after fertilization in different media.

Maturation Media	Fertilization Media	Numbers of Oocytes	Pronuclear Status (%) 1 PN	2PN	>2PN	Total Fert. (%)	UI (%)
TCM199	BO-Caff	41	18(43.9)	17(41.5)	2(4.9)	19(46.3)[a]	4(9.7)
	TALP-Caff	45	19(42.2)	18(40.0)	5(11.1)	23(51.1)[a]	3(6.7)
	CR1aa-Caff	44	17(38.7)	21(47.7)	3(6.8)	24(54.5)[a]	3(6.8)
CR1aa	BO-Caff	47	21(44.7)	23(48.9)	-	23(48.9)[a]	3(6.4)
	TALP-Caff	40	17(42.5)	19(47.5)	1(2.5)	20(50.0)[a]	3(7.5)
	CR1aa-Caff	49	14(28.6)	27(55.1)	4(8.1)	31(63.2)[b]	4(8.2)

Note : PN=Pronucleus; Total Fert.=Total fertilization; UI=Un-identified. Values within columns with the different superscripts are significantly difference (a-b, P<0.05).

Table 3. Cleavage rate and embryo development of zygote after cultured in different media

Culture Media	No. of Zygote	Development Stage (%) 2-8 cell	16-32 cell	Morula
TCM199	350	160(46.0)[a]	115(32.9)	98(28.0)[a]
CR1aa	420	236(56.2)[b]	168(40.1)	127(30.2)[b]

Values within columns with the different superscripts are significantly difference (a-b, P<0.05).

fertilization rate achieved in CR1aa-, BO- and Talp-media were 63.2%, 50.0% and 48.9%, respectively (Table 2, Figure 1B). The difference in the fertilization rate using different fertilization media (BO, TALP, CR1aa) was also presumed caused by the difference of energy resource existing in the three media. The energy source of the BO medium is derived from glucose and pyruvate, while in the TALP medium it is derived from pyruvate and lactate, and the energy source of CR1aa is derived from pyruvate, lactate, and amino acid. The use of glucose as an energy resource must initially be converted to lactate and pyruvate through the tricarboxylate acid cycle. Heparin is added in all media to be effective for *in vitro* capacitation of goat spermatozoa (Younis *et al.*, 1991). Heparin apparently binds to spermatozoa and plays a role in sperm uptake calcium (Parrish *et al.*, 1986). Caffeine acts synergistically with heparin to enhance efficacy of sperm (Niwa and Ohgoda, 1988). Caffeine enhances and pro-

longs sperm motility, even in bad-quality of sperm (Izquierdo *et al.*, 1998). However, the penetration rate of spermatozoa was still mainly depending on the high quality of the sperm used (Sumantri *et al.*, 1997).

The cleavage rate and embryo developed to morula of zygotes were higher (P<0.01) when cultured in CR1aa (56.2% and 30.2% for cleavage and morula stage, respectively) than in TCM-199 (46.0% and 28.0% for cleavage and morula stage, respectively) (Table 3, Figure 2). This may be due to the fact that in the CR1aa medium used for IVM/IVF/IVC, oocytes did not undergo change in their environment during the process of *in vitro* embryo production in CR1aa medium. The culture medium employed in IVM not only affects the proportion oocytes that reach metaphase II and become capable of undergoing fertilization, but can also influence subsequent embryonic development (Bavister *et al.*, 1992). The existence of the components within the CR1aa medium allows the embryo to pass the abstraction of embryo development. Glutamine metabolism in the early development of embryo is relatively high compared to morula and blastocyst phases. This clearly indicated that glutamine is said to be the energy resource in the early development of embryo. Besides, pyruvate and lactate existing in CR1aa medium can be used as the energy resources in the process of embryo development *in vitro*.

The present study shows that production of *in vitro* goat embryo could be produced by using CR1aa medium for maturation, fertilization and culture.

References

Bavister, BD., Rose-Hellekant, T.A., and Pinyopummintr, T., 1992. Development of *in vitro* matured *in vitro* fertilized bovine embryo into morulae and blastocyst in define culture media. *Theriodenology* 37;127-146.

Boediono, A., Takagi, M., Saha, S., and Suzuki, S. 1994. The influence of day 0 and day 7 superovulated cow serum during development of bovine oocytes *in vitro*. *Reprod. Fertil. Dev.* 6:261-264.

Boediono, A., Suzuki, T., and Godke R.A. 2003. Comparison of hybrid and pure-bred *in vitro*-derived cattle embryos during *in vitro* culture. *Anim. Reprod. Sci.* 78:1-11.

Brackett, B.G., and Oliphant, G. 1975. Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod.* 12:260-274.

Crozet, N., Huneau, D., De Smedth, V., Theron, M.C., Szollosi, D., Torres, S. and Sevellec, C. 1987. *In vitro* fertilization with normal development in the sheep. *Gamete Res.* 16: 159-170.

De Smedt, V., Crozet, N., Ahmed-Ali, M., Martino, A., and Y. Cognie. 1992. *In vitro* maturation and Fertilization of goat oocyte. *Theriogenology* 37:1049-1060.

Izquierdo, D., Villamediana, P., Palomo, M.J., Mogas, T. and Paramio, M.T. 1998. Effect of sperm capatation and fertilization media on IVF and early development of prepubertal goat oocytes. *Theriogenology*, 49:1501-1513.

Niwa, K. and Ohgada, O. 1988. Synergistic effect of caffeine and heparin on *in vitro* fertilization of cattle oocyte matured in culture. *Theriogenelogy* 30: 733-741.

Parrish, J.J., Susko-Parish J.L., Leibfriend-Rutledge M.L., Crister, E.S., Eyestone, W.H., and First, N.L. 1986. Bovine *in vitro* fertilization with frozen-thawed semen. *Theriogenology* 25: 591-600.

Pawshe, C.H., Totey, S.M. and Jain, S.K. 1994. A comparison of three methods of recovery of goat oocytes for *in vitro* maturation and fertilization. *Theriogenology* 42:117-125.

Pawshe, C.H., Palanisamy, A., Taneja, M., Jain, S.K. and Totey, S.M. 1996. Comparasion of various maturation treatments on *in vitro* maturation of goat oocyte and their early embryonic development and cell numbers. *Theriogenology* 46:971-982.

Rose, T.A., and Bavister, B.D. 1992. Effect of oocyte maturation medium on *in vitro*

development of *in vitro* fertilization bovine embryos. *Mol. Reprod. Dev.* 31:72-77.

Reiger, D. and Loskutoff, N.M. 1994. Changes in metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes *in vitro*. *J. Reprod. Fertil.* 100:257-202

Rosenkrans, C.F. and First, N.L. 1994. Effect of free amino acids and vitamins on cleavage and development rate of bovine zygote *in vitro*. *Anim. Sci.*, 72: 434-437.

Sumantri, C., Boediono, A., Ooe, M., Murakami, M., Saha, S. and Suzuki, T. 1997. The effect of sperm-oocyte incubation time on *in vitro* embryo development using sperm from a tetraparental chimeric bull. *Anim. Reprod. Sci.* 48:187-195.

Totey, S.M., Pawshe, C.H. and Singh, G.P. 1993. *In vitro* maturation and fertilization of buffalo oocytes *(Bubalus bubalis)* effect of media, hoemone and sera. *Theriogenology* 39:1153-117

Younis, A.I., Zuelke, K.A., Harper, K.M., Oliveira, M.A.I and Brackett, B.G. 1991. *In vitro* fertilization of goat oocytes. *Biol. Reprod.* 44: 1177-1182