IN VITRO DEVELOPMENT OF HOLSTEIN AND JAPANESE BLACK BREEDS EMBRYO

PERKEMBANGAN *IN VITRO* EMBRIO SAPI-SAPI HOLSTEIN DAN JAPANESE BLACK

A. BOEDIONO[1)] **and T. SUZUKI**[2)]

ABSTRACT

Two experiments were conducted with 3,457 oocytes, aspirated from the ovaries of slaughtered Holstein and Japanese Black breeds cows to compare the use of superovulated cow serum (SCS) and fetal calf serum (FCS) supplementation into medium for in *vitro* development (experiment I) and the effect of different source of oocytes (experiment II). The endpoint was development to cleavage on day 2 and to blastocyst up to day 9 after insemination. In experiment I, oocytes were matured and cultured *in vitro* (after fertilization) in TCM-199 supplemented with 5% SCS or 5% FCS. The cleavage rate of zygotes cultured in medium supplemented with 5% SCS was higher ($P<0,05$) than 5% FCS (5621860, **65,3%** vs 4451742, **60,0%**). The blastocyst production rate **was significantly** higher ($P<0,01$) in SCS treatment than FCS (2321860, **27,0%** vs 1241742, 16,7%). In experiment 11, 956 oocytes collected from Holstein breed and 899 oocytes from Japanese Black breed were matured, fertilized and cultured *in vitro* in medium supplemented with 5% SCS. The mean oocyte number and blastocyst production per *ovary* were **12,0** (956180) and 3,2 (252180) for Holstein breed, 15,0 (899160) and **3,1** (184160) for Japanese Black breed. The cleavage rate of zygotes derived from Holstein and Japanese Black breeds ovaries was not significantly different (6351956. **66,4%** vs 5691899, **63,3%,** respectively). However, the blastocyst production rate was significantly higher ($P<0,01$ in Holstein than Japanese Black breeds **(252/956, 26,4% vs** 1841899, **20%)**. These results indicated that the developmental rate to **blastocysts** of embryos cultured *in vitro* differ from breed to breed.

[1)] **Department of Anatomy, Faculty of Veterinary** Medicine, **Bogor Agricultural University, Jl. Taman Kencana 3, Bogor 16151, INDONESIA**

[2)] United **Graduate School of Veterinary** *Sciences* **Yamaguchi University, Yamaguchi 753,** JAPAN.

ABSTRAK

Telah dilakukan dua eksperimen dengan memakai 3.457 oosit yang diperoleh melalui aspirasi ovari sapi Holstein dan Japanese Black, untuk mengetahui pengaruh serum sapi superovulasi (SSS) dan Fetal Calf Serum (FCS) yang disuplementasikan ke dalam medium perkembangan *in vitro* (Eksperimen I) dan pengaruh sumber oosit yang berbeda (Eksperimen II). Pengamatan dilakukan terhadap perkembangan sampai tingkat pembelahan pada hari ke-2 dan tingkat blastosis pada hari ke-9 setelah inseminasi. Pada eksperimen 1, oosit dimatangkan dan dikultur *in vitro* (setelah dibuahi) dalam medium TCM 199 yang ditambah dengan 5% SSS atau 5% FCS. Rata-rata tingkat pembelahan sigot yang dikultur dalam medium diberi tambahan SSS 5% lebih tinggi (P<0,05) dibanding FCS 5% (5621860, 65,3% vs 4451742, 60.0%). Sedangkan rata-rata blastosis yang dihasilkan secara bermakna lebih tinggi (P<0,01) dalam SSS dibandingkan dengan FCS (2321860, 27,0% vs 1241742, 16,7%). Dalam eksperimen II, sebanyak 956 oosit yang diambil dari sapi Holstein dan 899 oosit dari Japanese Black dimatangkan, dibuahi dan dikultur secara *in vitro* dalarn medium yang diberi tambahan SSS 5%. Rata-rata jumlah oosit dan blastosis yang dihasilkan per ovari dari sapi Holstein adalah 12.0 (956180) dan 3,2 (252180): sedangkan dari Japanese Black adalah 15,0 (899160) dan 3.1 (184160). Rata-rata tingkat pembelahan zigot yang berasal dari ovari sapi Holstein dan Japanese Black tidak bermakna yaitu masing-masing 6351956, 66.4% dan 5691899, 63,3%. Namun demikian, rata-rata jumlah blastosis yang dihasilkan oleh ovari sapi Holstein secara nyata lebih tinggi (P<0,01) dibanding yang dihasilkan oleh ovari Japanese Black (252/956, 26,4% vs 1841899, 20,0%). Hal ini menunjukkan bahwa tingkat perkembangan embrio yang dikultur secara *in vitro* untuk mencapai tingkat blastosis berbeda dari satu jenis dengan yang lainnya.

INTRODUCTION

The developmental capacity of bovine oocytes after *in vitro* fertilization are influenced by factors such as the morphology of cumulus cells attached to oocytes, morphology of the ooplasm, the bull providing the semen and culture conditions (Shioya *et* al., 1988; Younis and Brackett, 1991; Kroetsch and Stubbings, 1992; Kajihara *et al.*, 1990; Boediono *et al.*, 1994). Normal embryonic development of *in vitro* matured and fertilized bovine oocytes has been achieved. Bovine oocytes matured with cumulus cells led to the production of zygote, and it develop, with subsequent pregnancies after transfer to recipient (Kajihara *et* al., 1990; Xu *et* al.. 1990).

Nuclear and extranuclear maturation events take place in vivo during follicular growth and ovulation. The changes go hand in hand with follicular maturation and are induced by characteristic changes in gonadotropic hormone profile. The presence of serum in culture medium was necessary for oocyte maturation, fertilization and culture since sera could provide cumulus oocyte complexes with protein requirements for *in vitro* maturation.

The purpose of the current study was to investigate the pre-implementation development of oocytes collected from Holstein and Japanese Black breeds after mature, fertilize and culture *in vitro* in medium supplemented with superovulated cow serum (SCS).

MATERIALS AND METHODS

Oocyte collection and in *vitro* maturation :

Cow's ovaries were obtained at a local slaughterhouse and were brought to laboratory within 3 h in physiological saline solution [0.9% (w/v) NaCl] supplemented with Penicillin-G (100 IU/ml) and streptomycin sulfate (0.2 mg/ml) at 30°-32°C. Oocytes in follicles 2-5 mm in diameter, were collected by aspiration with an 18-G needle filled on a 5 ml syringe in Modified-PBS. Only oocytes surrounded by cumulus cells over more than one-third of their surface and an evenly granulated cytoplasm were used in this experiment (Fig. 1A). Oocytes were then washed 2-3 times using maturation medium (TCM-199, Earle's salt. Gibco, Grand Island, NY, USA) supplemented with 0.01 mg/ml follicle stimulating hormone (FSH, Denka Pharmaceutical Co., Kawasaki. Japan), 50 μ g/ml gentamycin sulfate (Sigma Chemical Co., St. Louis MO.. USA) and 5% superovulated cow serum (SCS. collected on day-7 of estrus) or 5% fetal calf serum (FCS, Sigma Chemical Co., St. Louis MO., USA). The oocytes (100-200 oocytes) were then introduced into the maturation medium (2.5ml) in a polystyrene culture dish (35 mm diameter, Falcon 1008, Becton Dicknson Co. Ltd., Oxnard, CA., USA) covered with mineral oil (E.R. Squibb & Son, Inc., Princeton, NJ, USA) and cultured for 20 to 22 h at 38.5°C under 5% CO_2 in humidified air.

In *vitro* fertilization and culture :

Frozen-thawed semen from a single bull (Japanese Black breed) and the same lot was used for fertilization *in vitro*. Frozen sperm was thawed in water (30°-35°C), and the spermatozoa were diluted to about 6 ml with 2.5 mM caffein added to Brackett and Oliphant's medium (Caff-BO, Brackett and Oliphant, 1975) without bovine serum albumin and washed twice by centrifugation at 1800 rpm for 5 min each. The resultant sperm pellet was resuspended in Caff-BO supplemented with 0.3% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis MO., USA) and 20 μ g/ml heparin (Shimizu Pharmaceutical, Shimizu, Japan) to a final concentration of 5 x 10^6 spermatozoa per ml. A 100 μ 1 aliquot of the sperm suspension was covered with mineral oil and pre-incubated for 1 h under the condition described above. Oocytes matured in *vitro* (Gig. 1B) were transferred into sperm microdrops (20 to 25 oocytes per microdrop) for insemination (Fig. 1C).

After 5 h of insemination, oocytes with adherent cumulus cells were washed by repeated pipetting in culture medium and transferred to the culture medium (TCM-199) supplemented with 5 μ g/ml insulin (Wako Pure Chemical Industries. Osaka, Japan), 50 μ g/ml gentamycin sulfate and 5% SCS or 5% FCS for further development. The culture medium (0.5 ml) in a polystyrene dish (4-well multidish; Nuclon, Roskilde. Denmark) was covered with mineral oil (0.5 ml).

The cumulus cells surrounding the embryos were removed by pipetting after 48 h of insemination, but the cumulus cells layer (forming a monolayer at that time) attached to the bottom of culture dish were not disrupted and the embryos were cultured on this layer. The culture medium was replaced with new medium 4 days after insemination.

Experiment I :

During maturation and culture of bovine oocytes fertilized in vitro, the medium was supplemented with 5% SCS or 5% FCS. Each treatment was repeated four times, the sample of SCS and FCS remaining constant throughout.

Experiment II :

Oocytes of Holstein and Japanese Black breeds were collected separately. According to the results of experiment I. the maturation and culture medium in this experiment was supplemented with 5% SCS. The examinations were replicated 7 times using 956 Holstein oocytes and 899 Japanese Black oocytes

Embryo evaluation :

The proportions of embryos that had cleaved (2-. 4- and 8-cell stage) were recorded 48 h after insemination (when oocytes were taken from cumulus cells). Blastocyst development was assessed on day'-7, -8 and -9, when counted, blastocysts were removed from the culture dish to avoid double counting. The data were analyzed by Chi-square test. A probability of $P<0.05$ and $P<0.01$ were considered to be statistically significant.

Superovulated cow serum (SCS) collection :

The SCS was collected from superovulated cows treated with a total 20 mg FSH. Prostaglandin was administrated 48 h after FSH injection to induce luteolysis. Blood was collected on day-7 (on the time of embryo collection). Whole blood samples were centrifuged twice (3500 rpm, 10 min at 5°C). The serum obtained was then heat-inactivated (56°C, 30 min), distributed in small tube (2 ml) and kept in deep freezer (-20°C). The frozen serum were thawed before use.

RESULTS

Experiment I :

The cultured oocytes displayed a marked expansion of cumulus cells as characteristic for matured ova (Fig. 1B). Oocytes were evaluated 48 h after insemination, when developed to cleavage (2-, 4-, 8-cell stage, Fig. 2A), and when developed to blastocyst on day-7, -8 and -9 (Fig. 2C, 2D). The development rate of embryos cultured in medium supplemented with different sources of serum to cleavage rate of zygotes cultured in medium supplemented with 5% SCS was higher ($P<0.05$) than 5% FCS (5621860, 65.3% vs 4451742, 60.0%). The blastocyst productions rate was significantly higher ($P<0.01$) in SCS treatment than FCS (2321860, 27.0% vs 1241742, 16.7%.

Experiment II :

Total 956 oocytes collected from Holstein breed and 899 oocytes from Japanese Black breed were used for *in vitro* maturation, fertilization and culture. The development rate of Holstein and Japanese Black breeds *in vitro* produced embryo are shown in Table 2. The mean oocyte number and blastocyst production per ovary were 15.0 (956180) and 3.2 (252180) for Holstein breed, 15.0 (899160) and 3.1 (184160) for Japanese Black breed. The cleavage rate of zygotes derived from Holstein and Japanese Black breeds ovaries was not significantly different (6351956, 66.4% vs 5691899. 63.3%, respectively). However, the blastocyst production rate was significantly higher ($P<0.01$) in Holstein than Japanese Black breeds (256/956, 26.4% vs 184/899, 20%).

The development *in vitro* of Holstein and Japanese Black breeds embryos on day-2 (when it developed to 2-, 4-, and 8-cell stage), -7. -8, and -9 (when it developed to blastocyst) after insemination are shown in Figure 3.

DISCUSSION

Suzuki *et. al.* (1992) and Shioya *et. al.* (1988) reported that follicular cells, especially the cumulus cells surrounding immature oocytes, play central role in developmental competence in inducing not only nuclear maturation but also cytoplasmic maturation. Mucinate expansion of cumulus cells after *in vitro* maturation is a sign of nuclear and cytoplasmic maturation (Fig. 1B). When cumulus cells are removed, the zona pellucida hardens (Katska *et al.,* 1989) and this occurs under a prolonged culture period of germinal, vesicle-stage oocytes. The cumulus cells surrounding the oocytes and the present of serum in medium will protect the zona pellucida against hardeing. Following oocyte maturation in the absence of cumulus cells and/or serum, fertilizability is decreased in proportion to sperm penetration. The nutrients required for oocyte maturation are transported into the ooplasm via the junction gap between the ooplasm and cumulus cells used for *in vitro* fertilization a high number of the fertilized bovine oocyte cleaved and developed into blastocysts.

Co-culture with somatic cells is necessary for *in vitro* oocyte development to the blastocyst stage following fertilization. Fertilized oocytes have been co-cultured with bovine oviductal epithelial cells (BOEC; Durnford and Stubbings, 1992; Rorie *et al.*, 1992), granulosa cells (Mochizuki et *al.*, 1991), and cumulus cells (Kajihara *et al.*, 1990, Boediono *et al.*, 1994). In the present study, the embryos were co-cultured with cumulus cells for further development. As shown by Suzuki *et. al.* (1992) and Kajihara *et. al.* (1990), the co-culture of *in vitro* fertilized oocytes with cumulus cells provides an appropriate environment for embryonic development, and they obtained pregnancies and offspring by transfemng embryos resulting from co-culture with cumulus cells.

In early work hormonal and follicular factors were found to affect maturation of sheep oocytes *in vitro* and their subsequent developmental capacity (Moor and Trounson, 1977). In *vitro* matured oocytes in hormone free medium show a limited developmental competence (Hensleigh and Hunter. 1985). Several researcher used the medium supplemented with fetal calf serum (FCS; Saeki *et al.*. 1990), estrus cow serum (ECS; Schellander *et al.*, 1990), and superovulated cow serum (SCS: Boediono *et al.*. 1994: Suzuki *et al.*. 1992). When maturation and culture medium was supplemented with serum. a high proportion of the fertilized bovine oocytes developed into blastocysts. Suzuki et. *al.* (1982) reported that the medium supplemented with serum of the same species was having a beneficial effect for the survival of the embryo cultured *in vitro*. The results reported here demonstrated that SCS contains substances which increase the cleavage competence and developmental capacity into blastocysts of *in vitro* matured and fertilized bovine oocytes. This conclusion draws support from result vf Boediono *et al.* (1994) who used SCS for oocyte maturation, fertilization and culture of bovine oocytes, which developed into blastocysts.

The further development *in vitro* of Holstein and Japanese Black breeds fertilized oocytes to cleavage (2 to 8-cell stage) were not differ significantly. However, the blastocyst production rate was significantly higher in Holstein and Japanese Black breeds. McLaren and Bowman (1973) found that the difference in the start of cleavage in mouse was determined by maternal genotype In the present *study*, the maternal genotype had no affect on the cleavage rate of bovine oocyte. However, blastocyst rate produced from Holstein breed was higher than from Japanese Black breed. These results indicated that the developmental rate into blastocysts of embryos cultured in *vitro* differ from breed to breed.

ACKNOWLEDGMENTS

We would like to thank Dr. Nayeem, H.A.K and Dr. Khan, M.A. for critical reading of the manuscript, Kita Kyushu and Hiroshima slaughterhouse for the donation of the ovaries.

REFERENCES

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

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

Durnford. R. and Stubbings. R.B.. 1992. The influence of serum and oviductal cells during *in vitro* maturation on blastocyst development. Theriogenology. 37:205 abst.

Hensleigh. H.C. and Hunter, A.G., 1985. *In vitro* maturation of bovine cumulus enclosed primary oocytes and their subsequent *in vitro* fertilization and cleavage. J. Dairy Sci., 68:1456-1562.

Kajihara, Y.. Kometani, N., Kobayashi, S.. Shitanaka. Y.. Koshiba. Y. Hishiyama, K., Shiraiwa. K. and Goto, K., 1990. Pregnancy rates and births after co-culture of cumulus cells with bovine embryos derived from *in vitro* fertilization of *in vitro* matured follicular oocytes. Theriogenology. 33:202 abst.

Katska. L., Kauffold, P., Smorag, Z., Duschinski. U.. Torner. H. and Kanitz, W.. 1989. Influence of hardening of the zona pellucida on *in vitro* fertilization of bovine oocytes. Theriogenology, 32:767-777.

Kroetsch, T.G. and Stubbings, R.B.. 1992. Sire and insemination dose does effect in *vitro* fertilization on bovine oocytes. Theriogenology, 37:240 abst.

McLaren, A. and Bowman, P., 1973. Genetic effects on the timing of early development in the mouse. J. Embriol. Exp. Morphol. 30:491-498.

Mochizuki. H.. Fukui, Y. and Ono, H., 1991. Effect of number of granulosa cells added to culture media for *in vitro* maturation, fertilization and development of bovine oocytes. Theriogenology, 36:973-986.

Moor, R.M. and Trounson, A.O., 1977. Hormonal and follicular factors affecting maturation of sheep oocytes *in vitro* and their subsequent developmental capacity. J. Reprod. Fert., 49:101-109.

Rorie, R.W., Lester, T. D.. Miller, G.F. and Gliedt, D.L., 1992. *In vitro* development of bovine embryos co-cultured with bovine oviductal epithelial cells (BOEC) in either synthetic oviductal fluid (SOF) of M-199 medium. Theriogenology, 37:287 abst.

Saeki. K.. Leibfried. M.L. and First, N.L., 1990. Are fetal calf serum and hormone necessary during ***in vitro*** maturation of cattle oocytes for subsequent development?. Theriogenology, 33:316 abst.

Schellander, K., Fuhrer, R., Brackett, B.G., Korb, H. and Schleger, W., 1990. ***In vitro*** fertilization and cleavage of bovine oocytes matured in medium supplemented with estrous cow serum. Theriogenology, 33:477-485.

Shioya, Y., Kuwayama. M.. Fukushima, M. and Iwasaki, S., 1988. ***In vitro*** fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cells and matured ***in vitro.*** Theriogenology, 30:489-496.

Suzuki, T., Takahashi, Y., Shimohira, I., Oketani, R and Saito, N., 1982. Nonsurgically transfer and culture of bovine embryos ***in vitro*** in the medium supplemented with bovine serum collected from superovulated heifers 7 to 8 days after estrus. Jpn. J. Anim. Reprod., 28(3):119-123.

Suzuki, T., Singla, S.K., Sujata, J. and Madan, M.L. 1992. ***In vitro*** fertilization of water buffalo follicular oocytes and their ability to cleave ***in vitro.*** Theriogenology, 38:1187-1194.

Younis. A.I. and Brackett, B.G., 1991. Importance of cumulus cells and insemination interval for development of bovine oocytes into morulae and blastocysts ***in vitro.*** Theriogenology, 36:11-21.

Table 1. Development of IVF embryos cultured in *vitro* using different sera.

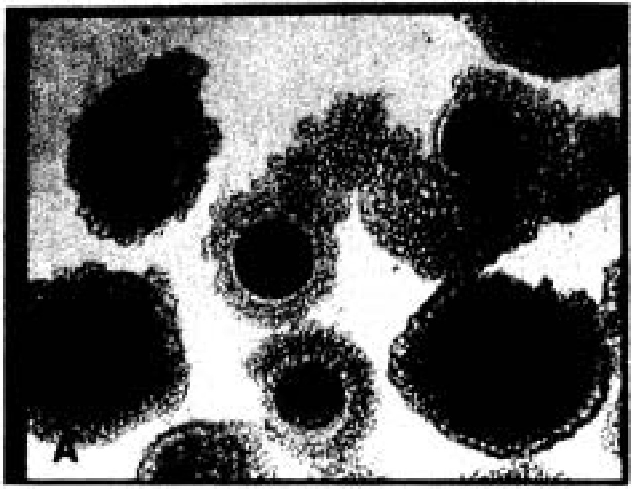
Serum	No.Oocytes assessed	No.of embryo cleaved				Blastocyst (%)
		2-cell	4-cell	8-cell	Total (%)	
SCS	860	42	146	374	562" (65.3)	232^c (27.0)
FCS	742	62	72	311	445" (60.0)	124^d (16.7)

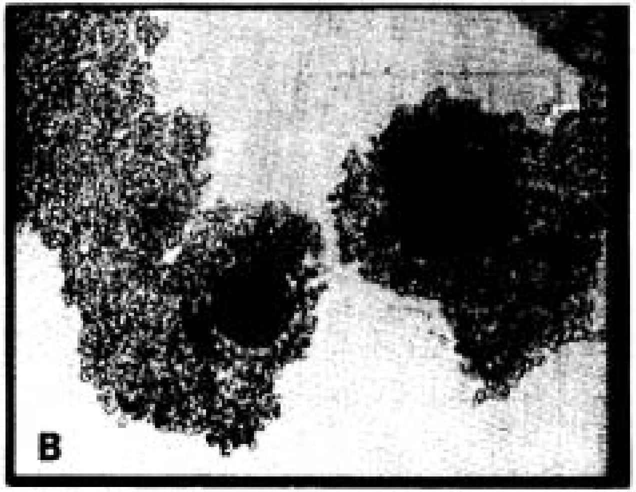
SCS – superovulated cow serum. FCS – fetal calf serum
within columns, means with different superscripts are significantly different (^{a,b} P 0.05; P 0.01, X^2-test).

Table 2. Development of Holstein and Japanese Black embryos in culture medium supplemented with superovulated cow serum.

Breed	No.ovary	No.oocytes	Developed to (%)	
			cleavage	blastocyst
Holstein	80	956	635 (66.4)	252 (26.4)"
Jpn. Black	60	899	569 (63.3)	184 (20.4)^b

^{a,b} within columns, means with different superscripts are significantly different (P 0.01, X^2-test).





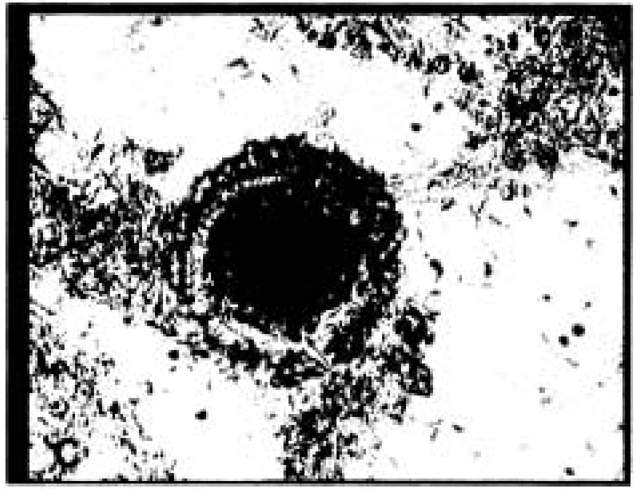


Figure 1. Maturation and **Fartilization** in *vitro* of Bovine **Oocvtes**

(A) Immature **oocytes, oocytes** surrounded by **cimmulus** cells and evenly **granulatted cytoplasm**

(B) **Matured oocytes, oocyrtes with** mucinate expansion of **cummulus** cells **after** 21 h cultured *in vitro*

(C) In vitro fertilization of matured **oocytes (with** I and II polar body present) by using capacitated sperm (I and **II** polar **body = arrow)**