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CO-CULTURE OF BOVINE EMBRYOS FROM OOCYTES MATURED AND FERTILIZED *IN VITRO* TO THE BLASTOCYST STAGE WITH OVIDUCTAL TISSUE*

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ABSTRACT

The development of in vitro *matured (IVM),* in vitro *fertilized (IVF) bovine embryos in co-culture with oviductal tissue suspension was investigated. Follicular oocytes (n=2929) collected from cattle at local slaughter houses were matured and fertilized* in vitro. *The follicular oocytes were graded according to their investments, i.e. compact, expanded, partially denuded and completely denuded as grades 1,2,3, and 4, respectively. Of 1134 ova in grade 1, 966 (85%) were penetrated by spermatozoa, fertilized and cleaved to a 2-cell stage at 30-48 h post insemination. In oocyte grades 2,3 and 4, 72%, 64% and 63% were observed respectively to be 2-cell. Bovine oviductal tissue culture was prepared by scraping luminal tissue of intact oviduct with a glass slide and suspended in TALP+10% heat-treated fetal calf serum. Zygotes were placed in treatment culture 18-22 h after insemination. Cleavage of the embryos up to the 6- to 8- cell stage were possible in all groups of oocytes. However, the development to late morula (LM) or blastocyst (BL) was observed only in the oocytes of grades 1 and 2. These results demonstrate that immature oocytes with cumulus cells can be matured and fertilized* in vitro, *and oviductal tissue co-culture can support normal development of IVM-IVF bovine embryo to LM and BL without using any intermediate recipients.*

INTRODUCTION

Embryo transfer (ET) has played a major role in cattle breeding during the past decade. The popularity of these techniques has created a significant need for a large number of embryos. It is generally accepted that *in vivo* conditions may offer a better environment for proper oocyte maturation than the *in vitro* counterpart and that a more physiological maturation renders the oocytes more reliable to undergo normal fertilization and embryo development. *In vivo* matured oocytes recovered from preovulatory follicles or flushed from

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the oviduct of superovulated cows are able to ensure full embryo development after *in vitro* fertilization (IVF) but the number of oocytes and calves born are still limited. The percentage of embryo development after successful IVF of *in vitro* matured eggs is even lower. Part of the problem stems from the existence of a block to *in vitro* culture at the 8-to 16-cell stage.[1-3] Nevertheless, the ability to mature oocytes *in vitro* from bovine ovary would greatly increase the yields of oocytes available for fertilization. Improved economics of embryo availability and production would largely contribute to the ET industry.

Attempts to improve the existing culture system by modifying composition or physical parameters have failed to yield consistent results. The absence of suitable *in vitro* methods has led many investigators to use ligated oviducts of rabbits[4,5] or sheep[6-9] as alternative systems for embryo culture.

In view of the fact that the oviductal environment of rabbit and sheep support early embryo development to the blastocyst stage, we hypothesized that a similar development could be achieved by co-culturing early bovine embryo with bovine oviductal tissue *in vitro.* The present study aims to investigate the developmental competence of *in vitro* maturation of oocytes fertilized *in vitro* and co-cultured with bovine oviductal tissue.

MATERIALS AND METHODS

Collection of oocytes

Bovine ovaries obtained from slaughter houses were transported to the laboratory in a thermos containing saline (25-30°C). Small antral follicles (1-5 mm in diameter) were aspirated with an 18- gauge needle and the contents pooled in 50-ml conical centrifuge tubes. The cumulus-oocyte complexes were recovered from the settled follicular fluid within 15-20 min under low power (20-30 x) stereomicroscope. Recovered oocytes were washed three times in HEPES-buffered Tyrode's media (TALP)[10] and oocytes were categorized on the basis of investment. Oocytes with intact, compact and unexpanded cumulus were classified as grade 1 whereas those with fully expanded, partially denuded and completely denuded were classified[11] as grades 2,3 and 4. Maturation *in vitro* was accomplished in TCM 199 modified with Earle's salt medium supplemented with 0.25 mM pyruvate, 10% heat-treated fetal calf serum (HTFCS), 15 μg/ml porcine follicle-stimulating hormone, 1 μg/ml LH and 1 μg/ml estradiol diluted with ethanol. Oocytes were then cultured in 60 mm Falcon dishes for 24 h at 39°C under an atmosphere of 5% CO_2, 95% air with high humidity. Maturation cultures were performed in 50 μl droplets (10 cumulus-oocyte complexes/droplet) under 10 ml paraffin oil in 60 mm petri dishes.

Sperm preparation

Ejaculated bovine sperms were obtained by artificial vaginal method at Pathumthanee Artificial Insemination Center and extended 1:1 with an egg yolk/sodium citrate extender to protect them during transit to the laboratory (1 h). The sperms were prepared using a method of motility enhancement called swim up. Sperms (0.25 ml) were layered under 1

ml of sperm-TALP-glucose free medium. After 1 h incubation at 39°C in a Forma Scientific incubator, the top portion (0.85 ml) of medium was removed from the tube. Separated sperms were centrifuged (200×g) for 10 min and the resulting sperm pellets were resuspended to 50×10^6 sperms/ml in sperm-TALP.

Isolation of oviductal tissue

Bovine oviducts of ovulated cows were obtained from slaughter houses and transported to the laboratory in 157 mM NaCl at 4°Ccontaining 50 μg/ml gentamicin. Then they were trimmed free of connective tissue and rinsed in 157 mM NaCl containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. After blotting on sterile gauze to remove excess fluid and blood, the oviducts were placed in sterile 100 mm petri dishes, grasped with a forceps at the isthmic end and scraped gently toward the infundibulum with a glass microslide. Mucosal tissue was then extruded from the ostium abdominale and transferred to a 12 ml conical centrifuge tube (Falcon 2095) with 10 ml TALP-HEPES supplemented with 10% HTFCS. The tissue was then washed in 5-7 changes of TALP-HEPES containing 10% HTFCS, 50 μg/ml gentamicin and then resuspended in TALP medium to a ratio of 1:50. Five milliliters of the suspension was placed in a 60 mm Falcon dish and cultured at 39°C under an atmosphere of 5% CO_2, 95% air. The cells were used for co-culture when they were above 80% confluency (day 2-7).

***In vitro* fertilization and co-culture**

A 10 μl aliquot (5×10^5 sperm/drop) of swim up separated sperms was placed in a culture dish with 50 μl of glucose free-TALP containing 0.2 μg/ml heparin to enhance capacitation. Sperms were incubated for 4h at 39°C in 5% CO_2 in air. Five *in vitro* matured cumulus-oocyte complexes were added to the drop. After 18–22 h of sperm : egg co-incubation, ova were removed from fertilization droplets and cumulus cells were stripped off by repeated pipetting through a small bore pipet. Then they were cultured in 10% HTFCS-TALP medium supplemented with oviductal tissue suspension (1:1) conducted in 50 μl droplets under paraffin oil (20 to 40 embryos/droplet). The medium was changed every 2 days.

Culture of embryos and assessment of development and viability

Embryos were assessed every day for cleavage using Nikon inverted microscope in a 39°C culture chamber. Eggs that were not penetrated after the first 24 h in culture as well as eggs penetrated but uncleaved were fixed and stained to ascertain whether fertilization had occurred. All eggs, except the uncleaved ones, were cultured for 10 days, and at the end of the designated culture period all embryos at LM and BL stages were frozen until embryo transfer was performed.

RESULTS

The competence of bovine embryo development in co-culture with oviductal cells was examined. Table 1 gives an overall view of the number of embryos which developed to various stages based on oocyte morphological gradings. Normal fertilization was defined as the number of oocytes showing two pronuclei (Fig. 1A). Compact oocytes (grade 1) had the highest frequency of normal fertilization compared with partial (grade 3) or denuded oocytes (grade 4). Of 1134 ova in grade 1, 966 (85%) were penetrated by spermatozoa, fertilized and cleaved to 2-cell embryos. In grades 2,3 and 4, 72%, 64% and 63% were observed respectively to be 2-cell at 30-48 h post-insemination. Development of morphologically normal 3-to 8-cell stage embryos varied among the 4 types of oocytes. However, the partially or completely denuded oocytes resulted in a lower percentage of development. Pronuclei, 2-,4-, 6- to 8- cell and 16- to 32- cell stages were observed at 18-22, 30-48, 42-60, 55-82, 75-115 h after *in vitro* insemination, respectively. The blastomeres were symmetrical (Fig.1 A-F). However, only the compact and expanded oocytes fertilized *in vitro* were developed to the morula and hatched blastocyst stages (Fig.1G-L) while the zygotes from partially or completely denuded ova cleaved to 2-cell through 8-cell stages but failed to form organized morulae or blastocysts. From a total of 1890 2-cell embryos matured from grades 1 and 2, after 75-115 h *in vitro,* 884 (47%) had cleaved regularly to the 16-to 32-cell stages. After culture for 96-150 h 666 embryos out of 1890 (35%) had developed to the morula stage and 386 (20%) had developed further to the blastocyst stage. Indeed, some of the blastocysts were maintained to day 12-14 in culture and hatched. *In vitro* viability was determined by the ability of culture to support development to the hatched blastocyst.

DISCUSSION

The present data show that co-culture of early bovine embryos with oviductal tissue can support normal development of early bovine embryos *in vitro* to LM and BL. Embryos held up to 14 days in co-culture hatched and developed, similar to those observed *in vivo.* The proportion of cleavage to LM or BL was higher in co-culture compared to using medium alone.[11] Most *in vitro* culture systems are unable to maintain development of embryos from early stages through hatching. Early bovine embryos cultured *in vitro* generally fail to develop beyond the 8- to 16-cell stage. This situation led Thibault[1] to suggest the existence of a block to *in vitro* development at the 8- to 16-cell stage. Blocks to *in vitro* development have also been described at the 2-cell stage in out-bred mouse,[12] hamster[13] and gerbil embryos,[14] 4-cell in porcine embryo[15,16] and 8-cell in ovine embryo.[17] However, of ovine embryos transferred to the environment of the oviduct, development from the 1-cell to blastocyst is possible. The data indicate that a high percentage of one-cell embryos cultured in this manner in both rabbit[4,5] and sheep[6,7] oviducts develop to the blastocyst stage.

Oviductal tissue has been successfully used as a supplement to *in vitro* embryo culture in early ovine embryos[17] but the work has been done with embryos from superovulation and *in vivo* fertilization. With respect to *in vitro* matured eggs and *in vitro* fertilization (IVM and IVF) in our experiments, the percentage of blastocysts obtained after co-culture

TABLE 1 *In vitro* oocyte maturation, fertilization and development of bovine embryos with oviductal cell culture. Classification was based on the morphology of bovine eggs (grade 1-4)

Cow oocytes grade	No. oocytes	No. replicates	Stages of Embryo Development					
			2-cell/ total ova (%)	3-4 cell/ 2-cell (%)	6-8 cell/ 2-cell (%)	16-32 cell/ 2-cell (%)	Morula/ 2-cell (%)	Blastocyst/ 2-cell (%)
1	1134	10	966 (85)	840 (87)	609 (63)	486 (50)	361 (37)	247 (26)
2	1281	10	924 (72)	798 (86)	588 (64)	398 (43)	305 (33)	139 (15)
3	294	10	189 (64)	126 (67)	84 (44)			
4	220	10	140 (63)	76 (54)	55 (39)			

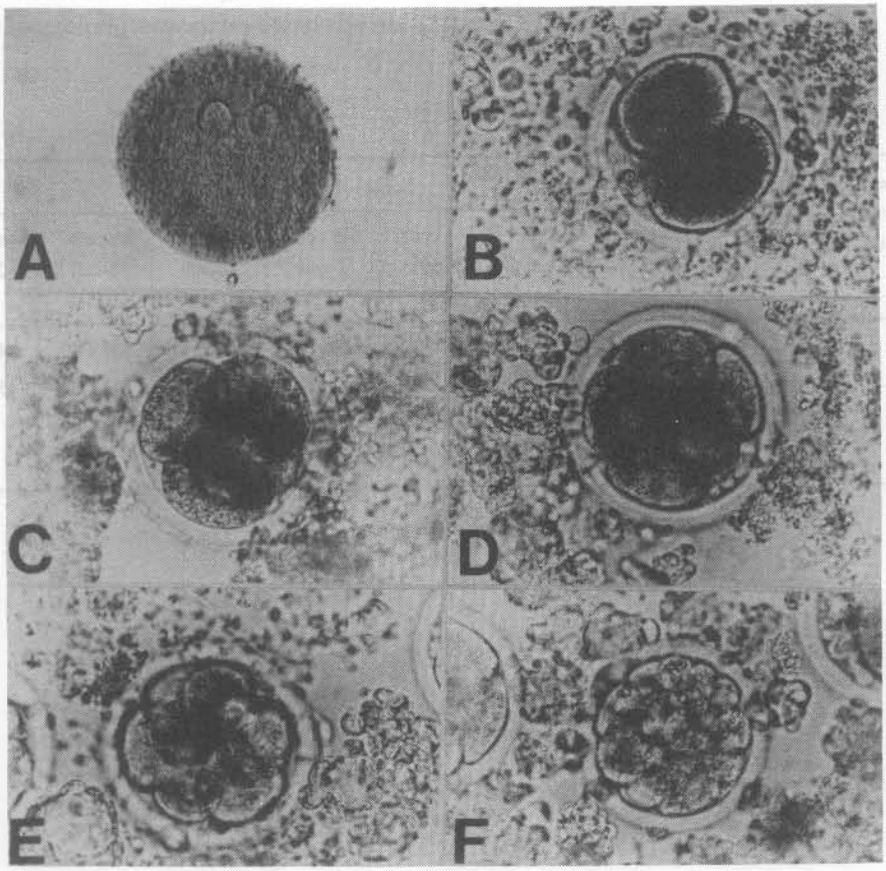

Fig. 1 Bovine embryos at various stages of development.

A. Two pronuclei stained with aceto-orcein after 18-hour post-insemination (hpi, x400).
B. 2-cell embryo, 33 hpi (x400)
C. 4-cell embryo, 45 hpi (x400)
D. 6-cell embryo, 58 hpi (x400)
E. 8-cell embryo, 65 hpi (x400)
F. More than 16-cell embryo, 86 hpi (x400)

Note that bovine embryos were co-cultured with oviductal cells from B to F.

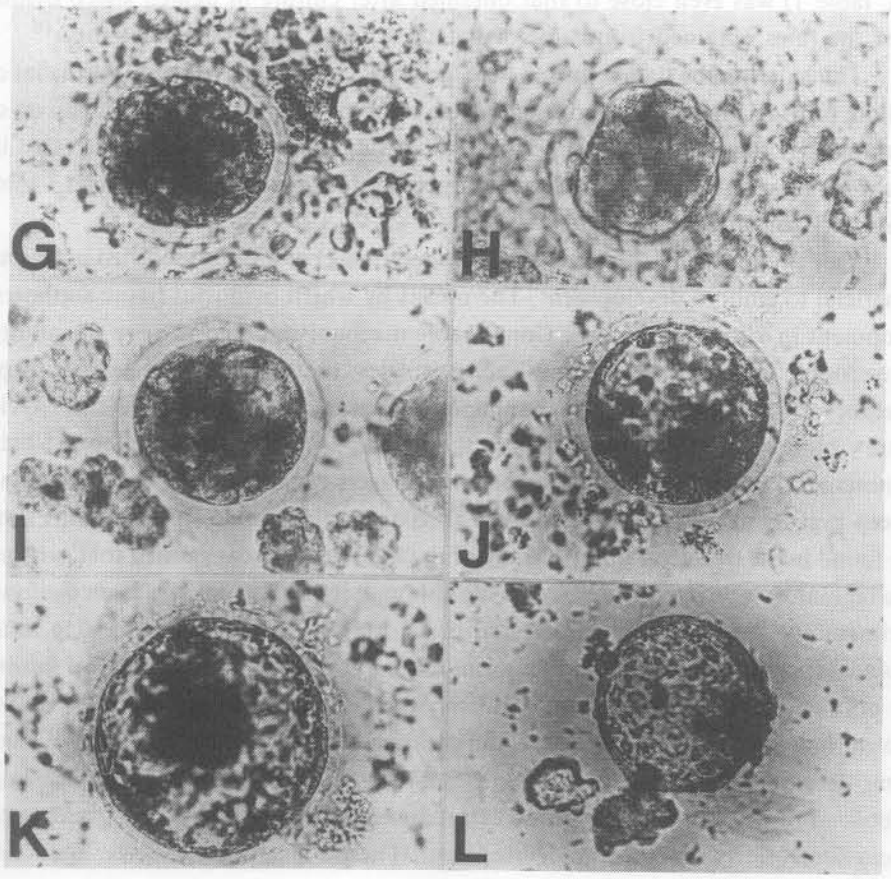

Fig. 1 (continued) Bovine embryo development from LM to hatched BL co-cultured with oviductal cells.

G. LM embryo, 120 hpi (x400)
H. Compact morula, 148 hpi (x400)
I. Early BL, 160 hpi (x400)
J. BL, 172 hpi (x400)
K. Fully expanded BL, 230 hpi (x400)
L. Hatched embryo, 280 hpi (x200)

LM = late morula BL = blastocyst

(35%, Table 1) was even close to that obtained after culture in ligated sheep oviduct[5] or half of the rates obtained from superovulated cattle on day 6-8 post estrous.[18]

Considering the data reported here, primary cultures of oviductal epithelial cells in co-culture provide a means of producing bovine embryos from IVM and IVF. This co-culture system for early stage embryos is superior to other successful culture techniques that involve medium alone or co-culture with other cell types such as trophoblastic vesicles,[3] fibroblast monolayers[19] or chicken amniotic fluid.[20]

Our findings indicate that the co-cultured tissue conditions modified in this manner is beneficial to embryo development. The means by which oviductal tissue supports early development *in vitro* is unknown. Conceivable mechanism could involve the addition of embryotrophic factors to the medium such as the low molecular weight peptide associated with embryotrophic fraction of medium conditioned by trophoblastic vesicles.[3] Oviductal secretory proteins have been described in the mouse,[21] rabbit,[22] sheep,[23] cow,[24] baboon[25] and human.[26] Furthermore, oviductal cell monolayers capable of supporting development of sheep zygotes to the blastocyst stage *in vitro* appear to secrete some proteins similar to those found in the oviductal fluid.[17] Many of these authors have suggested that such proteins play a regulatory role in early development, although in no case has this been demonstrated experimentally. Alternatively, co-cultured tissue may be involved in removing inhibitory substances including pyruvate and hypoxanthine. Pyruvate is known to be an inhibitor of early porcine embryo development[27] although it is required by early mouse embryos.[28] Hypoxanthine, a component of some complex media used for embryo culture can block murine development at the 2-cell stage.[29] Both hypoxanthine and pyruvate might be metabolized by the co-cultured tissue, their reduced concentrations thereby accounting for the beneficial effects observed on development. These substances, however, are not known to inhibit early bovine embryo development.

Due to its simplicity and economy, the co-culture system demonstrated in the present study offers an attractive alternative to *in vivo* culture in the ligated oviducts of sheep or rabbits. Moreover, elucidation of the mechanism underlying the positive influence of co-culture is expected to lead to the development of a defined media for bovine embryos.

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บทคัดย่อ

ได้ทำการเพาะเลี้ยงตัวอ่อนโคในหลอดทดลอง โดยใช้เซลล์ไข่ซึ่งเจริญเติบโตและปฏิสนธิกับเซลล์อสุจิในหลอดทดลอง จากนั้นทำการเพาะเลี้ยงร่วมกับเซลล์เยื่อบุท่อนำรังไข่ของโค ได้แบ่งเซลล์ไข่จำนวน 2929 เซลล์ ซึ่งทำการเพาะเลี้ยงให้เจริญเติบโตในหลอดทดลองออกเป็น 4 ประเภท โดยใช้เซลล์หุ้มเซลล์ไข่เป็นเกณฑ์ เซลล์ไข่ที่มีเซลล์หุ้มหนาแน่น (ประเภท 1) เมื่อได้รับการปฏิสนธิกับเซลล์อสุจิ 30-48 ชั่วโมง จะแบ่งตัวได้เป็นตัวอ่อนระยะ 2 เซลล์ ถึง 85% (966/1134) ส่วนเซลล์ไข่ประเภท 2,3 และ 4 ภายหลังการปฏิสนธิได้ตัวอ่อนระยะ 2 เซลล์ 72% 64% และ 63% ตามลำดับ เตรียมเซลล์บุท่อนำรังไข่โดยขูดท่อนำรังไข่ และนำมาเพาะเลี้ยงในน้ำยาเพาะเลี้ยง ภายหลังการปฏิสนธิ 18-22 ชั่วโมง นำตัวอ่อนมาเพาะเลี้ยงร่วมกับเซลล์บุท่อนำรังไข่ ตัวอ่อนจากทั้ง 4 กลุ่มสามารถแบ่งไปถึงระยะ 6-8 เซลล์ได้ แต่ตัวอ่อนในกลุ่มที่ 1 และ 2 สามารถแบ่งตัวต่อไปจนถึงระยะมอรูลาหรือบลาสโตซีส จากผลการวิจัยครั้งนี้แสดงให้เห็นว่าเซลล์ไข่ที่มีเซลล์หุ้มสมบูรณ์สามารถเจริญเติบโตและปฏิสนธิในหลอดทดลอง พร้อมทั้งสามารถเพาะเลี้ยงร่วมกับเซลล์ท่อนำรังไข่จนถึงระยะที่จะทำการย้ายฝากตัวอ่อนได้ โดยไม่ต้องฝากถ่ายในสัตว์ทดลอง