Effect of Insulin and Basic Fibroblast Growth Factor (bFGF) on Bovine Embryo Development In Vitro in a Defined Medium after In Vitro Fertilization

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Abstract: *Present experiments were carried out to evaluate positive effects of growth factors and conditioned medium (CM) from bovine granulosa cells (BGC) in a defined medium on bovine embryo development. In Experiment 1, insulin (19%; blastocyst / oocytes examined) and basic fibroblast growth factor (bFGF; 14%) individually stimulated embryonic development to blastocyst stage in a defined medium as effective as serum did (15%; p>0.05), but neither bovine lipoprotein (LP; 3%) nor bovine serum albumin (BSA; 1%) affected the stimulation of embryo development. In Experiment 2, denuded embryos did not traverse beyond 8- to 16-cell block without the co-culture with BGC, regardless of the addition of insulin and bFGF. In Experiment 3, serum-free, CM of BGC in the presence of insulin stimulated blastocyst formation (11%) as well as co-culture system (18%). These results suggest that insulin and bFGF stimulate early bovine embryonic development through the action of a certain embryotrophic factor produced by the activation of the physiological function of BGC.*

Key words: *Cattle, Serum-free, Insulin, bFGF, Conditioned medium.*

In most mammalian species, early embryos cultured *in vitro* are arrested their development at certain stage in culture. This *in vitro* developmental blocks have been reported at 2-cell stage in mouse and hamster, 4-cell stage in pig, and 8-cell stage in sheep [1]. In cattle, development of early embryos is generally arrested the 8- to 16-cell stage [2].

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Successful culture of bovine embryos through the 8- to 16-cell block has required the use of intermediate hosts, such as the ligated rabbit oviduct [3], or sheep oviduct [4]. However, these techniques are too complicated to manipulate early embryos and to study physiological processes during embryonic development.

There have been recent reports in domestic animals that increased rate of early embryonic development has been obtained by co-culture with oviductal epithelial cells [5–10], trophoblastic vesicles [11] and granulosa cells [12–15] or other cell types [7]. For studying the mechanisms of somatic cell-embryo interactions, Eyestone and First [16] revealed that the oviduct cell-CM facilitates the bovine embryos development to morulae and blastocyst as well as that of co-culture system, suggesting that oviduct epithelial cells may release embryotrophic factor(s) to medium, or remove inhibitory substance(s) to embryo development. For the study of embryogenesis at the molecular level it is important to isolate and characterize the positive or negative factors of early embryo development derived from somatic cells.

We therefore attempted 1) to develop a completely defined medium which consistently allow development to blastocyst stage of bovine early embryo matured and fertilized *in vitro*, 2) to evaluate the effect of insulin, bFGF, BSA and LP on embryonic development with or without the co-culture of BGC and 3) to examine the embryotrophic ability of medium conditioned with BGC in a defined medium. Possible mechanisms concerning the *in vitro* developmental blocks of the bovine embryo were discussed.

Materials and Methods

Collection and in vitro maturation of oocytes: Ovaries were obtained from Japanese black cows and heifers killed at a local slaughter house. The ovaries were brought to the laboratory within 4 h, then washed three times with phosphate-buffered saline (PBS) and cut into two pieces with scissors. After further washing with PBS, the divided ovaries were placed into 100 mm culture dishes with 20 ml TCM199 (Nissui) supplemented with 25 mM HEPES and without sodium bicarbonate (pH 7.4), containing 100 μg/ml of polyvinylalcohol (PVA; Sigma), 1.25 mM sodium pyruvate, 15 μg/ml heparin (Sigma) and 10 μg/ml gentamycin (m-TCM199). After that, the divided ovaries were minced with a surgical knife and the small vesicular follicles were ruptured. Only oocytes with an intact, unexpanded cumulus oophorus were selected for experiment. The selected cumulus-oocyte complexes (COC) were rinsed twice with m-TCM199 and rerinsed with the maturation medium described below. Then, 25 to 30 oocytes were transferred into 0.35 ml of TCM199 without HEPES and with 10% (v/v) heat-inactivated fetal calf serum (FCS; Lot. 910, Cat. 29-101-54R, Flow) covered with mineral oil (Squibb), and cultured in a CO_2 incubator (5% CO_2, 95% air with high humidity at 38.5 °C) for 21–23 h.

Sperm preparation and Insemination: A frozen straw of semen was thawed in warm water (32–35 °C) and an aliquot of semen was transferred into BSA-free BO medium [17] containing 5 mM caffeine (Wako) and with 15 μg/ml heparin. Spermatozoa contained in the aliquots of semen were washed twice by centrifugation at 500 × g for 7 min and were resuspended in the same medium. The spermatozoa concentration was adjusted to be approximately 1.0×10^7/ml. For *in vitro* fertilization, a 50 μl of spermatozoa suspension was added to the equal volume of BO medium containing 10 mg/ml BSA (A-7511, Sigma) and 5 mM caffeine. Each droplet contained 25–30 COC matured *in vitro*.

In vitro development: After 6 h of insemination, all inseminated oocytes were washed and transferred into 0.35 ml each of the various development media tested and cultured for further development in a CO_2 incubator. All culture dishes were pre-treated with TCM199 containing 0.15 mg/ml collagen (Type 1-A; Nitta gelatin) for 1 h at room temperature for coating the dishes.

Embryos were denuded from surrounded BGC by gentle pipetting at 48 h (Exp. 1) and 24 h (Exps. 2, 3) after insemination, and the denuded embryos were co-cultured with the monolayer of BGC remaining in fresh development medium, or were cultured alone in a fresh development medium.

Experiment 1: This experiment was performed to evaluate factors which stimulate embryonic development in a defined medium co-cultured with BGC. Inseminated oocytes were cultured in TCM199 supplemented with (1) TCM199 alone, (2) 5 μg/ml insulin (I-5500, Sigma), (3) 10 ng/ml bFGF (R&D Systems), (4) 25 μg/ml bovine lipoprotein (LP; EX-CYTE), (5) 1 mg/ml BSA (A-7511, Sigma), (6) four factors included (4F) and (7) 5% FCS. Furthermore, dishes were pre-coated with collagen, and all media include 0.5 μg/ml aprotinin (A-4529, Sigma), protease inhibitor, and media were changed every 2 days. The experiment was done in 4 replicates.

Experiment 2: This experiment was performed to determine whether insulin or bFGF in defined medium could directly stimulate embryonic development to blastocyst stage without the co-culture of BGC or not. Since some of embryos developed beyond 8-cell stage at 48 h post-insemination, removal of cumulus/granulosa cells surrounding embryos were done at 24 h post-insemination. After the cumulus/granulosa cells surrounding embryos were completely removed by using Pasteur pipettes, the denuded embryos were washed three times with a TCM199 medium. These denuded embryos were incubated in (1) TCM199 alone, (2) TCM199 supplemented with 5 μg/ml insulin, (3) TCM199 supplemented with 10 ng/ml bFGF, and or (4) co-cultured with BGC in TCM199 supplemented with 5 μg/ml insulin. The experiment was done in 3 replicates.

Experiment 3: The objective of this experiment is to evaluate the embryotrophic activity in a conditioned medium of BGC cultured in TCM199 supplemented with 5 μg/ml insulin and 0.5 μg/ml aprotinin. Conditioned medium was collected as follows: COC were placed into a 25-cm^2 flask (MS20050; Sumitomo) in a growth medium consisting of an equal volume mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F12; D8900, Sigma) supplemented with 10% FCS, and then cultured in a CO_2 incubator until the primary culture of BGC became confluent. The confluent culture of BGC were washed twice with PBS and dissociated with 0.025% trypsin-0.02% EDTA. The cell suspensions were centrifuged at 400 × g for 5 min. The cell pellet was suspended in medium DMEM/F12 containing 10% FCS into 75-cm^2 flask (MS20250; Sumitomo). At confluency, stock cultures were harvested with trypsin-EDTA and the cells were split at a ratio of 1:4 for inoculation into new culture flasks. Each harvest and split was referred to as one passage. When the cells at a passage four

reached confluence, the cells washed twice with 10 ml of PBS and then 8 ml of fresh defined medium (TCM199 supplemented with 5 μg/ml insulin and 0.5 μg/ml aprotinin) was added to the culture flasks. The CM was collected every 48 h, and centrifuged at 800 × g for 10 min to get rid of cell debris. The developmental ability of embryos in this CM was assessed in comparison with those in co-culture system and fresh TCM199 medium alone. The experiment was done in 4 replicates.

Statistical analyses: Statistical analyses in our study were carried out by the chi-square test for the comparison of the frequencies of development.

Result

Experiment 1: The effects of insulin, bFGF, LP and BSA on the embryonic development in a defined medium co-cultured with BGC were shown in Table 1. While the cleavage rates (2-cell / oocytes) did not vary among culture conditions, the proportions of embryo development to blastocyst stage (blastocyst / oocyte, blastocyst / 2-cell) were significantly ($p<0.01$) increased by the addition of insulin or bFGF and combination of four factors (4P) in comparison with that in TCM199 medium alone. The greatest rate of embryos developed to blastocysts was obtained in TCM199 supplemented with insulin, however, did not show significant difference ($p>0.05$) between TCM199+insulin and serum-supplemented medium (19% vs 14%). The addition of LP and BSA did not stimulate the embryonic development ($p>0.05$).

Experiment 2: The effects of insulin and bFGF on embryonic development of denuded embryos without co-culture with BGC were shown in Table 2. Although the rate of cleavage (>2-cell) and 8-cell stage did not differ ($p>0.05$) among different culture conditions, but no further development of denuded embryos cultured in TCM199 with insulin or bFGF and/or TCM199 alone was observed. In contrast, high frequency ($p<0.01$; 20%) of denuded embryos developed to blastocyst stage was obtained in a co-cultured system with BGC.

Experiment 3: The proportion of blastocyst formation was higher in CM than in fresh medium ($p<0.01$; 11% vs 4%, Table 3). The rate of blastocyst cultured in CM was nearly equal to that in co-culture ($p>0.01$, 11% vs 18%, respectively), although the rate of blastocyst per cleaved embryos in CM was lower than that in co-culture with BGC ($p<0.01$; 16% vs 32%).

Discussion

In this study, when embryos were co-cultured with bovine BGC in a chemically defined medium, insulin

Table 1. The effects of various growth factors on bovine embryo development to the blastocyst stage in a defined medium *in vitro*

Culture conditions		No. of oocytes examined	No. and % developed to: >2-cell	>8-cell	>Blastocyst	Blastocyst / >2-cell
TCM199	alone	131	75 (57%)	44 (34%)	5 (4%)[a]	7%[a]
	+Insulin	133	79 (59%)	58 (44%)[a]	25 (19%)[b]	32%[b]
	+ bFGF	118	69 (58%)	50 (42%)	17 (14%)[b]	25%[b]
	+LP	91	55 (60%)	38 (42%)	3 (3%)[a]	5%[a]
	+BSA	86	44 (51%)	27 (31%)	1 (1%)[a]	2%[a]
	+4F	87	46 (53%)	30 (34%)	13 (15%)[b]	28%[b]
TCM199	+5% FCS	170	76 (45%)	48 (28%)[b]	24 (14%)[b]	32%[b]

a vs b: Means with different superscripts in the same column differ ($p<0.01$).

Table 2. Development of denuded embryos cultured in TCM199 supplemented with insulin or b-FGF and/or co-culture with granulosa cells

Culture condition			No. of oocytes	No. and % developed to: >2-cell	>8-cell	>Blastocyst
Medium		Denudation				
TCM199	alone	Denuded	59	37 (63%)	25 (42%)	0 (0%)[a]
	+insulin	Denuded	269	153 (57%)	108 (40%)	0 (0%)[a]
	+b-FGF	Denuded	56	34 (61%)	21 (38%)	0 (0%)[a]
TCM199	+insulin	Co-culture	282	177 (63%)	132 (47%)	56 (20%)[b]

a vs b: Means with different superscripts in the same column differ ($p<0.001$).

Table 3. Development of bovine embryos after fertilization *in vitro* and culture in a serum-free conditioned medium with bovine granulosa cells

Culture system	No. of oocytes examined	No. and % developed to: >2-cell	>8-cell	>Blastocyst	Blastocyst />2-cell
Co-culture	214	122 (57%)	88 (41%)	39 (18%)[a]	32%[A]
CM	201	135 (67%)[a]	108 (54%)[a]	22 (11%)[a]	16%[B]
Fresh medium	174	88 (51%)[b]	63 (36%)[b]	0 (0%)[b]	0%[C]

a, b, A–C: Means with different superscripts in the same column differ ($p<0.01$).

and bFGF independently promoted development of 1-cell bovine embryos through 8- to 16-cell stage to blastocyst stages with high frequency as serum did. Furthermore, serum-free BGC-CM was nearly effective as the co-culture with BGC in supporting embryo development *in vitro*.

It has been reported that the presence of granulosa cells was important for normal fertilization and further development in rabbit [18], sheep [19], and cow [20] in serum-containing medium. Our recent findings indicated that cell proliferation of cumulus and/or BGC surrounding with early bovine embryos might be primarily important to increase the embryo development *in vitro* in a serum-free medium [21]. Moreover, insulin and bFGF are potent mitogens for granulosa cells in serum-containing [21] and serum-free media [23–25]. Insulin is well established to modulate the cell function of GC such as steroids and oxytocin production which are directly involved in follicular and oocytes maturation [26–28]. Moreover, it has been accumulating evidences that insulin acts directly on the embryo development *in vitro* in some species, e.g., 1) increase rat embryo development [29], 2) stimulates the DNA, RNA, and protein synthesis on mouse embryos *in vitro* [30, 31]. And mouse and bovine embryos have specific binding sites for insulin [31, 32]. In addition, Shamsuddin *et al.* [33] revealed that bovine IVM-IVF zygotes developed up to blastocyst stage without co-culture with somatic cells under serum-free semi-defined conditions, such as with BSA, insulin, transferrin and selenium. However, our observation (Experiment 2) demonstrated that denuded 1-cell embryos failed to proceed to blastocyst stages without co-culture with BGC. These results suggest that insulin may have synergistic effect with other promoting factor(s) rather than direct effect on the stimulation of bovine embryo development *in vitro*.

Basic FGF is present in wide variety of tissue source and exhibit a deverse physiological activities including cell proliferation and cell differentiation, tissue formation and regeneration [34]. While bFGF is a principal mitogens for GC *in vitro*, it acts as potent inhibitor of maturation of immatured GC *in vitro* by supporting LH/hCG receptor induction and progesterone secretion [35]. The gene expression of bFGF in BGC [36] indicates that bFGF may act as an autocrine regulator of GC functions. Present study revealed that bFGF only stimulated bovine embryo development beyond 8- to 16-cell block when these embryos were co-cultured with BGC, but no blastocyst formation has been observed in BGC-free culture system.

Lipoproteins and BSA also stimulated the proliferation of GC in a serum-free medium [23–25]. Bovine lipoprotein (LP) used in this study is an aqueous mixture of lipoproteins (mostly high density lipoprotein and minor contaminant of low density lipoprotein) which bind cholesterol, phospholipids and unsaturated fatty acids. BSA is a common protein supplement in both somatic cell culture and embryo culture media in place of serum. Commercial BSA contains many kinds of substances like lipid-soluble fatty acids, steroid hormones, and undefined other serum components [37–39]. Recent studies [26, 28] demonstrated that lipoprotein may play an important role for follicular and oocytes maturation by affecting the steroidogenesis of GC. Moreover, Rizzino [40] observed that lipoprotein directly stimulated the outgrowth of mouse embryos cultured in serum-free medium as extensive as that observed in serum-supplemented medium. As to the effect of BSA, there are a few reports [41, 42] that fertilization of rat oocytes following maturation has significantly increased in BSA-containing medium. On the other hand, BSA did not support viability or maturation of bovine cumulus-oocytes *in vitro* [43, 44]. Therefore, the stimulatory effect of BSA on embryo development is controversial. Our experiment presented that lipoprotein and BSA failed to increase the proportion of bovine blastocysts formation *in vitro* in a defined medium whereas embryos were co-cultured with BGC. The possible explanation of this phenomena is that neither lipoprotein nor BSA supported the viability of BGC in TCM199 medium alone, but insu-

lin and bFGF were able to maintain the long-term survival of these cells at the same culture conditions.

In Experiment 3, BGC-CM in a defined medium (TCM199 supplemented with only 5 μg/ml insulin) is capable of supporting bovine embryonic development beyond 8- to 16-cell stage. As insulin has shown no direct effect on bovine embryo development without the co-culture with BGC (Experiment 2), it may be considered to induce the production of a certain embryotrophic factor by BGC. However, the proportion of blastocysts per cleaved embryos in CM was significantly lower than that in co-culture with BGC. This indicates that the presence of viable cells may be necessary to ensure a continuous supply. It has been reported that the CM with oviduct epithelial cells [9, 16, 45], and trophoblastic vesicles [11] promoted bovine embryo development to morulae and blastocyst stage *in vitro*. Further investigation is required to characterize whether these factors, which can be synthesized and secreted by BGC, are identical with factors from oviductal epithelial cells and trophoblastic vesicles or not.

In conclusion, insulin and bFGF independently stimulated bovine embryonic development to blastocyst stage with the co-culture of bovine BGC in a serum-free, defined medium. The rate of blastocysts induced by insulin or bFGF were as equal as that in serum-containing medium. Furthermore, BGC-CM collected in the presence of insulin allowed the denuded embryos to traverse beyond 8- to 16-cell block, and this result suggests BGC may synthesize and secrete embryotrophic factor in the presence of insulin.

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ウシ体外受精卵の体外発生における Insulin および basic Fibroblast Growth Factor (bFGF) の影響

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牛体外受精胚の体外発生における細胞成長因子と顆粒膜細胞培養上清液の効果について無血清培地を用いて検討した．実験1では，顆粒膜細胞との共培養系において lipoprotein (3%; 胚盤胞/卵胞卵子) および BSA (1%) は牛初期胚の発生を促進しなかったが，insulin (19%) および線維芽細胞成長因子 (bFGF; 14%) はそれぞれ血清添加培地 (15%) と同様に胚発生を促進することが明らかとなった．しかし，これらの因子は顆粒膜細胞を除去した場合には全く効果を示さなかった (実験2)．また，顆粒膜細胞の無血清培養上清液 (11%) に共培養 (18%) と同様の胚発生促進作用が認められた (実験3)．以上の結果から，insulin と bFGF は顆粒膜細胞に作用し，間接的に牛初期胚の発生を促進することが示された．

キーワード：ウシ，無血清培地，Insulin，bFGH，培養上清液．