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Interaction between embryos and culture conditions during *in vitro* development of bovine early embryos

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Summary

Various factors such as embryo density and substances in the medium can influence embryo development *in vitro*. These factors and the embryos probably interact with each other, however the interactions are not fully understood. To investigate the interactions, we examined the effects of the number of embryos, drop size, oxygen concentration and glucose and inorganic phosphate in the medium during protein-free culture of bovine IVM/IVF embryos. In Experiment 1, different numbers of embryos were cultured in a 50 μl drop of medium. The frequencies of blastocyst development in the groups with 25, 50 and 100 embryos per drop were higher than in the other groups. One, five and 25 embryos were cultured in different drop sizes (Experiment 2), a 50 μl drop of medium at different O_2 concentrations (Experiment 3) and a 50 μl drop of medium excluding glucose and/or inorganic phosphate (Experiment 4). In Experiment 2, the size of the medium drops did not improve blastocyst development. In Experiment 3, the highest frequency of blastocyst development for one, five and 25 embryos per drop was obtained at 1, 2.5 and 5% O_2, respectively. In Experiment 4, blastocyst development for one and five embryos per drop were improved in the medium excluded inorganic phosphate. These results indicate that there is a cooperative interaction among embryos during culture and that this interaction may be mediated by reduction of toxic factors in the medium. At low embryo density, reduced oxygen concentration or the exclusion of inorganic phosphate enhanced blastocyst development.

Keywords: Bovine embryo, Culture medium, Density, Development, Oxygen

Introduction

Recently, many successful *in vitro* culture systems for bovine early embryos have been reported and applied to many reproductive technologies. However, the rates of development to the blastocyst stage, which is the critical point to term, remain at around 20–30% of cultured embryos (Lee *et al.*, 2004; Fujita *et al.*, 2006; Lim *et al.*, 2006). It has been reported that the early development of mammalian embryos is greatly suppressed in cultures with single or reduced numbers of embryos (Lane & Gardner, 1992; Kato *et al.*, 1994; Ikeda *et al.*, 1999; Mizushima & Fukui, 2000; Fujita *et al.*, 2006). These results indicate that the culture system of early embryos is inadequate and incomplete. There must be some limiting factors in the *in vitro* embryo culture systems.

It has been suggested that suppression of early development in individual cultures might be caused by a deficiency of cooperative interaction among embryos (Keefer *et al.*, 1990; Paria & Dey, 1990; O'Neill, 1997). Autocrine factors such as epidermal growth factor, platelet-activating factors, insulin-like growth factors, etc., in culture drops have been shown to affect embryonic development (Yang *et al.*, 1993; O'Neill, 1997; Gopichandran & Leese, 2006). However, the mechanisms of the interactions remain unclear. Discovering the mechanisms of the interactions might make it possible to establish a completely controlled culture system and also make the culture of fewer numbers of embryos derived from individual cattle by ovum pick-up, nuclear transfer and transgenesis

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successful. Chemically defined media have been developed for the culture of bovine zygotes to the blastocyst stage (Pinyopummintr & Bavister, 1991; Nagao *et al.*, 1994). Defined media facilitate the analytical study of beneficial or suppressive factors in embryo development and the mechanisms of early development of bovine embryos under controlled environmental conditions.

Co-culture with various somatic cells has been shown to support bovine embryonic development from the 1-cell stage to the blastocyst stage (Eyestone *et al.*, 1989; Goto *et al.*, 1989). We previously reported that one of the beneficial roles of bovine oviductal epithelial tissue during co-culture with bovine early embryos may be to remove toxic substances from the medium (Nagao *et al.*, 1994). Therefore, there may be a cooperative interaction among bovine early embryos and culture conditions mediated by the reduction of toxic factors in the medium such as reactive oxygen (Nagao *et al.*, 1994), glucose and inorganic phosphate (Sirard *et al.*, 1988; Seshagiri & Bavister, 1989; Petters *et al.*, 1990). However, serum albumin or serum, which was commonly used as a component of culture media, may make it difficult to study the cooperative interaction, as these supplements contain unknown factors that influence embryonic development (Kane & Headon, 1980; Kane, 1983). Protein-free culture systems should facilitate study of the cooperative interaction.

In this study, we examined interactions between the number of embryos, drop size, oxygen concentration and presence of glucose and inorganic phosphate in the medium during development of bovine IVM/IVF embryos in a protein-free medium.

Materials and methods

Bovine oocyte collection and *in vitro* maturation

Oocyte collection and *in vitro* maturation of the oocytes were carried out as previously described (Saeki *et al.*, 1990a). Briefly, bovine cumulus–oocyte complexes (COCs) obtained from antral follicles of slaughterhouse ovaries were washed and cultured for 21 h in tissue culture medium 199 with a 25 mM HEPES buffer (Cat. No. 12340, Gibco) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA; M.W. 30,000–70,000; Sigma Chemical Co.), 0.5 mM sodium pyruvate (Nacalai Tesque Inc.), 1% (v/v) antibiotic–antimycotic solution (Gibco), 0.02 AU/ml follicle stimulating hormone (Antrin) and 1 μg/ml estradiol-17β (Sigma).

In vitro fertilization

In vitro fertilization was carried out as described by Nagao *et al.* (1995b). Briefly, frozen–thawed semen was washed with a discontinuous Percoll solution (Saeki *et al.*; Pharmacia). The matured COCs (10 COCs/drop) were inseminated with the washed spermatozoa (1×10^6 cells/ml) in a defined medium (Brackett & Oliphant, 1975) modified by excluding glucose and supplemented with 1 μg/ml heparin (from porcine intestinal mucosa, Sigma; m-DM) in 100 μl drops of the medium covered with mineral oil (Squibb) in each culture dish (Falcon) for 6 h. Following the insemination, the sperm and cumulus cells were completely removed from the oocytes by vortex agitation (Sirard *et al.*, 1988). Some oocytes were fixed and stained for assessment of the fertilization (Iritani & Niwa, 1977).

In vitro culture of IVM/IVF embryos

In vitro culture of IVM/IVF embryos was essentially carried out as described by Nagao *et al.* (1995a). Briefly, denuded embryos which were 6 h post insemination were cultured in a protein-free modified synthetic oviduct fluid (m-SOF; Nagao *et al.*, 1995a) supplemented with or without glucose and/or phosphate (KH_2PO_4) under 5% CO_2, 5% O_2 and 90% N_2 at 39°C with high humidity. Aliquots (1–250) of the embryos were placed in droplets (1–250 μl) of the medium covered with mineral oil (Squibb) in each culture dish (Falcon). Both cleavage (≥2-cell) and development to the blastocyst stage were examined under a stereo-microscope (×60) 7 days following the insemination. Some blastocysts were fixed and stained immediately for determination of the cell number (Saeki *et al.*, 1990b).

Experimental design

Experiment 1. To examine the effects of the number of embryos, different numbers of embryos (1, 5, 10, 25, 50, 100 and 250) were cultured in 50 μl drops of the medium.

Experiment 2. To examine the effects of embryo density, one, five and 25 embryos were cultured in different sizes of the medium drop (1, 10, 50 and 250 μl).

Experiment 3. To examine the effects of the oxygen concentration and embryo number, one, five and 25 embryos were cultured in 50 μl drops of the medium at 1, 2.5, 5, 10 and 20% O_2 concentration.

Experiment 4. To examine the effects of glucose and inorganic phosphate in a culture medium and embryo number, one, five and 25 embryos were cultured in 50 μl drops of the medium excluding glucose and/or inorganic phosphate (KH_2PO_4).

Statistical analysis

The frequencies of cleavage and development to the blastocyst stage calculated for every replicate were

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Table 1 Effects of embryo number per drop in *in vitro* culture of bovine embryos on development to the blastocyst stage[a]

Embyro no. per 50 µl drop	No. of embryos cultured	No. (%)[b,c] of embryos cleaved	No. (%)[b,c] of blastocysts	No. of cells[c] per blastocyst
1	250	182 (72 ± 14)	27 (12 ± 1)[d]	95 ± 8[d]
5	250	193 (76 ± 7)	58 (20 ± 3)[e]	111 ± 8[d,e]
10	250	192 (75 ± 8)	59 (24 ± 3)[e]	124 ± 7[d,e,f]
25	400	292 (71 ± 4)	117 (31 ± 5)[f]	134 ± 7[f]
50	400	304 (74 ± 7)	125 (30 ± 5)[f]	126 ± 7[e,f]
100	500	288 (72 ± 5)	129 (32 ± 7)[f]	115 ± 8[d,e,f]
250	1250	748 (75 ± 2)	202 (20 ± 1)[e]	106 ± 9[d,e]

[a]Replicate number of each experimental groups was five.
[b]Percentage of cultured embryos.
[c]Mean ± SEM.
[d,e,f]Values with different superscripts within the same column differ significantly ($p < 0.05$).

summarized as the mean ± SEM. The frequency and cell count of blastocysts were compared using Fisher's protected least significant difference (PLSD) test following analysis of variance (ANOVA).

Results

Experiment 1

The number of embryos in a drop during *in vitro* culture seriously affects early development of the bovine embryos. The frequencies of blastocyst development of groups of 25, 50 and 100 embryos/drop were higher than the other groups ($p < 0.05$, Table 1).

Experiment 2

When embryos were cultured in a 1 µl drop, cleavage and blastocyst development were suppressed irrespective of the embryo number/drop ($p < 0.05$, Table 2). In each number of embryos per drop, reducing or increasing the size of the drops (10, 50 and 250 µl drops) had no effect on the blastocyst development ($p > 0.05$).

Experiment 3

The highest frequencies of the blastocyst development in the group of one embryo per drop was obtained at 1% O_2, for five embryos in a drop group it was at 2.5% O_2 and for 25 embryos in a drop group it was at 5% O_2 ($p < 0.05$, Table 3). Reduced oxygen concentration improved blastocyst development of fewer number of embryos/drop.

Experiment 4

The frequencies of blastocyst development in groups of one and five embryos/drop were improved in

Table 2 Effects of embryo number and drop size in *in vitro* culture of bovine embryos on development to the blastocyst stage[a]

Culture condition				
Embryo no. per 50 µl drop	Drop size	No. of embryos cultured	No. (%)[b] of embryos cleaved	No. (%)[b] of blastocysts
1	1	65	48 (73 ± 4)	4 (6 ± 6)
	10	67	53 (83 ± 7)	5 (7 ± 2)
	50	67	56 (85 ± 4)	7 (11 ± 3)
	250	66	53 (82 ± 3)	4 (8 ± 4)
5	1	130	86 (64 ± 5)[c]	10 (7 ± 3)[c]
	10	140	116 (88 ± 3)[d]	35 (27 ± 3)[d]
	50	140	108 (79 ± 4)[d]	32 (23 ± 1)[d,e]
	250	140	109 (79 ± 3)[d]	27 (18 ± 3)[c,d,e]
25	1	125	77 (66 ± 10)[c]	7 (6 ± 2)[c]
	10	125	109 (89 ± 3)[d]	40 (36 ± 3)[d]
	50	125	102 (80 ± 4)	43 (34 ± 3)[d]
	250	125	99 (77 ± 4)	35 (28 ± 9)[d]

[a]Replicate number of each experimental groups was four.
[b]Percentage of cultured embryos were showed as mean ± SEM.
[c,d,e]Values with different superscripts within the same column and same group differ significantly ($p < 0.05$).

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Table 3 Effects of embryo number and oxygen concentration in *in vitro* culture of bovine embryos on development to the blastocyst stage[a]

Culture condition				
Embryo no. per 50 µl drop	Oxygen concentration (%)	No. of embryos cultured	No. (%)[b] of embryos cleaved	No. (%)[b] of blastocysts
1	1	96	79 (82 ± 3)	32 (35 ± 5)[c]
	2.5	100	80 (83 ± 4)	24 (24 ± 3)[d]
	5	100	83 (83 ± 3)	15 (12 ± 3)[e]
	10	100	81 (81 ± 3)	12 (12 ± 4)[e]
	20	85	68 (81 ± 3)	0 (0 ± 0)[f]
5	1	110	94 (85 ± 1)	30 (23 ± 5)[c]
	2.5	110	92 (85 ± 2)	50 (39 ± 8)[d]
	5	105	80 (76 ± 11)	29 (25 ± 2)[c]
	10	110	90 (82 ± 1)	26 (23 ± 3)[c]
	20	100	71 (74 ± 5)	6 (7 ± 2)[e]
25	1	100	47 (47 ± 8)[c]	8 (7 ± 2)[c]
	2.5	100	86 (78 ± 6)[d]	35 (31 ± 1)[d]
	5	100	88 (88 ± 5)[d]	50 (42 ± 5)[e]
	10	100	85 (85 ± 2)[d]	34 (33 ± 2)[d]
	20	100	85 (85 ± 3)[d]	16 (16 ± 2)[c]

[a]Replicate number of each experimental groups was four.
[b]Percentage of cultured embryos were showed as mean ± SEM.
[c–f]Values with different superscripts within the same column and same group differ significantly ($p < 0.05$).

Table 4 Effects of embryo number and medium composition in *in vitro* culture of bovine embryos on development to the blastocyst stage[a]

Culture condition					
	Medium composition				
Embryo no. per 50 µl drop	Glucose	Phosphate	No. of embryos cultured	No. (%)[b] of embryos cleaved	No. (%)[b] of blastocysts
1	–	–	79	73 (92 ± 1)[c]	10 (13 ± 3)[c]
	–	+	79	73 (93 ± 2)[c]	11 (13 ± 4)[c]
	+	–	82	62 (76 ± 2)[d]	19 (23 ± 2)[d]
	+	+	78	64 (82 ± 2)[e]	9 (11 ± 1)[c]
5	–	–	175	155 (86 ± 4)	44 (25 ± 5)[c]
	–	+	175	154 (88 ± 3)	56 (31 ± 4)[c,d]
	+	–	175	154 (87 ± 2)	73 (43 ± 6)[d]
	+	+	175	157 (89 ± 4)	49 (29 ± 4)[c]
25	–	–	150	126 (80 ± 4)	46 (30 ± 5)[c]
	–	+	150	125 (79 ± 2)	52 (34 ± 5)[c,d]
	+	–	150	131 (85 ± 3)	54 (35 ± 5)[c,d]
	+	+	150	134 (83 ± 3)	65 (45 ± 4)[d]

[a]Replicate number of each experimental groups was five.
[b]Percentage of cultured embryos were showed as mean ± SEM.
[c,d,e]Values with different superscripts within the same column and same group differ significantly ($p < 0.05$).

the medium which excluded inorganic phosphate ($p < 0.05$, Table 4). In a group of 25 embryos/drop, excluding glucose and/or inorganic phosphate had no effect on the blastocyst development ($p > 0.05$).

Discussion

Effects of the number of embryos during *in vitro* culture on the early development of bovine embryos were investigated in Experiment 1. We found that culturing embryos in groups (25, 50 and 100 embryos/drop) enhanced blastocyst development (Table 1), as has been shown in previous reports in mice (Paria & Dey, 1990; Lane & Gardner, 1992; Kato & Tsunoda, 1994) and cattle (Keefer *et al.*, 1990). This result shows the existence of a cooperative interaction among the embryos in a culture drop. The possible reasons for this effect are as follows: bovine embryos improve their developmental

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environment by producing factors that stimulate their own early development in culture and/or by reducing the levels of toxic factors in the medium. Co-culture of normal mouse embryos with uncleaved embryos has been shown to decrease the developmental ability of normal embryos (Salahuddin *et al.*, 1995). However, in our embryo culture condition, in which the average cleavage rate was around 70–80%, uncleaved embryos in culture drops did not affect the early development of fertilized embryos to blastocysts (data not shown).

The ligands and receptor mRNAs for many kinds of growth factors have been detected in the early embryos of many species (Rappolee *et al.*, 1988; Corps *et al.*, 1990; Watson *et al.*, 1992). It has been shown that growth factors are produced by early embryos as well as by oviduct epithelial cells and uterus epithelial cells and that supplementation of the growth factors clearly plays an important role in early embryonic development *in vitro* (Gardner & Kaye, 1991; Paria & Dey, 1993; Yang *et al.*, 1993: O'Neill, 1997: Gopichandran & Leese, 2006). These reports may be evidence of an autocrine role for growth factors during embryo development and suggest that decreasing the embryo number in culture drops will dilute the concentration of some growth factors in the drop, resulting in the suppression of embryo development. Therefore, we tried to improve blastocyst development in culture with fewer embryos by decreasing the drop volume, i.e. by concentrating some autocrine factors in the medium (Experiment 2). However, the volume of the medium drops did not affect blastocyst development (Table 2). This result indicates that the quantity of some of the growth factors produced by embryos might be very small and that the effects of these growth factors on development might be very slight. Alternatively, decreasing the drop volume may increase the concentration of metabolites such as ammonium (Orsi & Leese, 2004), resulting in a toxic developmental environment for the embryos.

We also speculated that bovine embryos may decrease the concentration of toxic factors in the drops, resulting in enhanced blastocyst development. In Experiment 3, we examined the relationship between the number of embryos and the oxygen concentration during culture. We found that oxygen concentration was correlated with embryo number and that lower oxygen concentrations enhanced the development of smaller numbers of embryos. The frequencies of blastocyst development for one, five and 25 embryos in a drop were highest at 1, 2.5 and 5% O_2, respectively (Table 3). A possible reason for this effect may be the consumption of oxygen in the medium by the aerobic metabolism of each embryo. The oxygen consumption of early mouse embryos has been shown to be around $150–500 \times 10^6$ µl/embryo/h (Mills & Brinster, 1967; Houghton *et al.*, 1996). Therefore, the oxygen concentration of the microenvironment around the embryos in microdrops may be reduced to a lower level than that of the gas phase in the incubator. A low oxygen concentration of around 5–10% has been shown to enhance early embryonic development in a cell-free group culture (Quinn & Harlow, 1978; Thompson *et al.*, 1990; Nagao *et al.*, 1994). Oxygen tension in the oviduct is approximately 11–60 mmHg (1.5–8.7%) (Mastroianni & Jones, 1965; Mass *et al.*, 1976; Fisher & Bavister, 1993). However, as shown in this study (Table 3), the optimal oxygen concentration for a single embryo in an *in vitro* cell-free condition may be lower than that previously reported. It has been previously shown that concentrations of reactive oxygen species in embryos increase proportionally to oxygen concentration, thereby causing lipid peroxidation and enzyme inactivation that result in cell damage (Nasr-Esfhani *et al.*, 1990; Ribarov & Benov, 1981).

We also examined the relationship between the number of embryos and glucose and inorganic phosphate (KH_2PO_4) in the culture medium in Experiment 4. We found that the amount of inorganic phosphate in the medium was correlated to embryo number and that excluding phosphate from the medium enhanced the development of fewer embryos (Table 4). Inorganic phosphate and glucose in the medium have been shown to be detrimental to embryo development (Schini & Bavister, 1988). Seshagiri and Bavister (1989) showed that inorganic phosphate regulates the glucose-mediated inhibition of development of hamster 8-cell embryos *in vitro*. These inhibitory effects were also observed in a pig embryo culture (Petters *et al.*, 1990). Therefore, 1.17 mmol inorganic phosphate in the medium may be detrimental for the early development of low density mbovine embryos as shown in Table 4.

Our results indicate that there is a cooperative interaction among bovine early embryos during *in vitro* culture and that this interaction may be mediated though the reduction of toxic factor(s). At low embryo density (one to five embryos/drop), reduced oxygen concentration (1–2.5%) or the exclusion of inorganic phosphate from the medium enhances blastocyst development. Our protein-free culture system for bovine embryos reveals these interactions first. Elucidating the mechanisms of these interactions might make it possible to establish a completely controlled culture system and also allow the culture of fewer embryos. These results may also be useful for practical and biological application of ovum pick-up, nuclear transfer and transgenesis culture systems for individual cattle.

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