Zygote **12** (May), pp. 143–150. © 2004 Cambridge University Press
DOI:10.1017/S0967199404002710 Printed in the United Kingdom

Additional effect of epidermal growth factor during *in vitro* maturation for individual bovine oocytes using a chemically defined medium

Takahiro Oyamada, Hiroshi Iwayama and Yutaka Fukui

Laboratory of Animal Reproduction, Department of Animal Production Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan

Date submitted: 13.05.03. Date accepted: 19.06.03

Summary

This study was performed to establish an individual bovine oocyte-IVP system using a chemically defined simple medium (mSOFaa containing 1 mg/ml polyvinyl alcohol: PVA) and to investigate the effects of epidermal growth factor (EGF) during oocyte maturation on *in vitro* maturation, fertilization and embryonic development. Cumulus–oocyte complexes were collected from bovine ovaries and were matured in mSOFaa containing PVA (control medium) supplemented with 0, 1, 10 or 50 ng/ml of EGF. Two further groups (TCM199 and mSOFaa, supplemented with 10% fetal calf serum were also included. In this study, mSOFaa containing PVA were used as a basic medium for fertilization and embryo development *in vitro*. Experiments were conducted in both group- and individual-IVP systems. In the group-IVP system, the proportion of matured oocytes (MII) in the control medium (62.7% ± 5.0%) was significantly ($p < 0.05$) lower than in all other treatments, and in the individual-IVP system, the addition of 1 ng/ml EGF significantly ($p < 0.05$) increased the maturation rate (1 ng/ml EGF vs control: 76.2% ± 5.4% vs 57.1% ± 14.4%). The addition of EGF did not affect the proportions of penetrated and normally fertilized oocytes in either individual- or group-culture systems. In the group-IVP system, no significant difference among treatments was found in the rate of blastocyst formation, whereas in the individual-IVP system the control medium supplemented with 10 ng/ml EGF resulted in a significantly ($p < 0.05$) higher the rate of blastocyst formation (20.0 ± 5.2%) than that in the control medium (6.2% ± 3.5%). These results indicate that bovine oocytes can successfully develop to blastocysts in an individual-IVP system using a single chemically defined medium, and that the group-IVP system also resulted in a similar level of blastocyst formation to that in a standard multiple-media system in our laboratory. The effect of EGF during oocyte maturation medium differed depending on whether embryos were cultured individually or in groups.

Keywords: Bovine, Defined medium, EGF, Individual culture, Oocyte

Introduction

Oocytes or embryos are usually incubated in groups during *in vitro* maturation (IVM), fertilization (IVF) and embryonic development (IVC) (group-IVP system). However, in the group-IVP system, oocytes aspirated from all visible follicles including healthy, growing and atretic follicles are pooled for IVM, and the developmental competence of individual oocytes is not assessed following IVF and IVC. If an individual-IVP system is established using a defined, protein-free synthetic culture medium, more detailed data could be obtained during oocyte maturation and embryonic development compared with the group-IVP system. Limited experimental samples, such as an oocyte from valuable livestock or a wild species, ovum pick-up and, in particular, micromanipulative techniques such as nuclear transfer, may also require an individual-IVP system. There are several studies describing

All correspondence to: Y. Fukui, Laboratory of Animal Reproduction, Department of Animal Production Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan. Tel: + 81 155 49 5415. Fax: + 81 155 49 5593. e-mail: fukui@obihiro.ac.jp

https://doi.org/10.1017/S0967199404002710 Published online by Cambridge University Press

individual-IVP systems (Keefer *et al.*, 1994; Thibodeaux *et al.*, 1995; Berthelot & Terqui, 1996; Araki *et al.*, 1997; Carolan *et al.*, 1996; O'Doherty *et al.*, 1997; Hagemann *et al.*, 1998, 1999; Hendriksen *et al.*, 1999); however, these systems involved addition of sera or bovine serum albumin (BSA), or a co-culture system was used. Furthermore, these studies showed that a large variation in the success of embryonic development and that an individual-IVP system is associated with low developmental competence to the blastocyst stage. Such exogenous proteins may introduce many unknown components into the culture environment and may add to the variability in results between and within laboratories (Kane, 1983; Keskintepe & Brackett, 1996). Fukui *et al.* (2000) and Mizushima & Fukui (2001) established an individual-IVP system using a semi-defined or defined medium containing BSA and polyvinyl alcohol (PVA), respectively.

Generally, different media have been used for each phase of *in vitro* embryo production, such as TCM199 for *in vitro* maturation, Tyrode's ALP for IVF, and either TCM199 or SOF for embryo culture. However, a single medium throughout all IVP steps may simplify the process. Gandhi *et al.* (2000) reported that a single medium system using mSOF containing BSA resulted in an equivalent level of embryonic development compared with the conventional approach. To our knowledge, attempts at an individual-IVP system using a single chemically defined, protein-free medium have not been reported.

Epidermal growth factor (EGF) has been found to positively influence *in vitro* maturation of various mammalian oocytes. The addition of EGF to maturation medium improves cumulus cell expansion (Lonergan *et al.*, 1996), oocyte maturation (Lonergan *et al.*, 1996; Anas *et al.*, 1998, 2000*a*), normal fertilization rate (Anas *et al.*, 2000*b*) and developmental competence (Lonergan *et al.*, 1996; Abeydeera *et al.*, 2000; Anas *et al.*, 2000*b*; Watson *et al.*, 2000). Anas *et al.* (2000*a*, *b*) suggested a novel physiological role for EGF in the cytoplasmic maturation of bovine oocytes. EGF specifically binds to the receptor that has tyrosine kinase activity, which has a critical role for maturation of mammalian oocytes. Watson *et al.* (2000) suggested that EGF supplementation of oocyte maturation medium resulted in a concentration-dependent increase in embryonic development. It would be necessary to clarify whether or not the benefical effect of EGF is the same between an individual-IVP system and a group-IVP system, and also to determine an appropriate dose of EGF in both systems.

The objectives of this study, therefore, were (1) to establish a bovine individual-IVP system using a single, simple defined medium (mSOF + PVA), and (2) to investigate the effect of adding different doses of EGF to the IVM medium on maturation, fertilization and embryonic development in bovine individual- and group-IVP systems.

Materials and methods

Oocyte collection and *in vitro* maturation (IVM)

Ovaries from Holstein and Japanese black heifers and cows were collected at a local abattoir shortly after slaughter and transported in physiological saline at 20 °C to the laboratory within 1 day, because of the disease control for bovine spongiform encephalopathy (BSE). The ovaries were washed twice in a fresh saline. Antral follicles (2–8 mm in diameter) were aspirated using an 18-gauge needle attached to a 5 ml disposable syringe. Aspirated cumulus–oocyte complexes (COCs) with compact intact cells and homogeneous cytoplasm were selected and washed in SOF-HEPES (see Table 1) supplemented with 1 mg/ml PVA (Sigma Chemical, St Louis, MO). Oocytes were placed in a drop of each maturation medium (group: 5 or 6 oocytes per 50 µl drop; individual: 1 oocyte per 10 µl drop). In addition, 0.02 IU/ml of porcine pituitary FSH (Antrin, Denka Chemical, Kawasaki, Japan) and 1 µg/ml estradiol-17β (Sigma, USA) were added to all maturation media. The oocytes were cultured for 22–24 h at 39 °C in an atmosphere of 5% CO_2 in air and 95% humidity.

Sperm preparation and *in vitro* fertilization (IVF)

A frozen straw of semen from a Japanese black bull was thawed at 37 °C then centrifuged (500 *g*) for 3 min. An aliquot of centrifuged semen was incubated with 2 ml of SOF-Cap (see Table 1) supplemented with 1 mg/ml PVA for a 1 h swim-up procedure (Fukui, 1990; Parrish *et al.*, 1986), from which the top 1.6–1.8 ml of the medium was collected and further centrifuged (500 *g*) for 10 min to remove any remaining semen extender. After IVM, oocytes were washed three times in PVA containing SOF-HEPES. For group-IVP system, 5 or 6 oocytes were placed in a 46 µl drop of SOF-IVF (see Table 1) supplemented with 1 mg/ml PVA, and 4 µl sperm suspension (final concentration in drop: 1×10^6 spermatozoa/ml) was added to each drop. For the individual-IVP system, each oocyte was placed in an 8 µl drop of SOF-IVF supplemented with 1 mg/ml PVA, and 2 µl sperm suspension (final concentration in drop: 1×10^6 spermatozoa/ml) was added to each drop. Oocytes were co-incubated with spermatozoa for 18–22 h at 39 °C in an atmosphere of 5% CO_2 in air and 95% humidity.

In vitro culture (IVC)

After insemination, oocytes were denuded of cumulus cells and residual spermatozoa by repeated pipetting

https://doi.org/10.1017/S0967199404002710 Published online by Cambridge University Press

Table 1 Composition of modified synthetic oviductal fluid (mSOF) used for maturation, fertilization and development of bovine oocytes *in vitro*

Reagent (mmol/l; mM)	SOF-IVM (IVM medium)	SOF-IVF (IVF medium)	SOF-Cap (Capacitation)	SOF-IVC (IVC medium)	SOF-HEPES (Washing)
Glucose	1.5	–	–	1.5	1.5
Glutamine	1	–	–	1	–
$NaHCO_3$	25	25	25	25	4
HEPES	–	–	5.0	–	10.5
$CaCl_2$	1.71	1.71	–	1.71	1.71
MEM, EAA[a]	2% (v/v)	–	–	2% (v/v)	2% (v/v)
MEM, NEAA[b]	1% (v/v)	1% (v/v)	–	1% (v/v)	1% (v/v)
Tri-sodium citrate	–	–	–	0.34	–
Myo-inositol	–	–	–	2.77	–
β-ME[c]	5 μM	–	–	–	–
Ascorbic acid	–	–	–	100 mg/ml	–
Heparin	–	5 IU/ml	–	–	–
Penicillamine	–	20 μM	20 μM	–	–
Hypotaurine	–	10 μM	10 μM	–	–
Epinephrine	–	2 μM	2 μM	–	–

[a] Eagle's minimum essential medium essential amino acids (Life Technologies, Grand Island, NY).
[b] Eagle's minimum essential medium nonessential amino acids (Life Technologies).
[c] β-mercaptoethanol.

and vortexing for a few minutes in 1 ml SOF-HEPES (Ca^{2+}, Mg^{2+}-free) containing 1 mg/ml hyaluronidase (Sigma, USA). Oocytes were then cultured in drops of SOF-IVC (see Table 1) supplemented 1 mg/ml PVA (group: 5 or 6 oocytes per 25 μl drop; individual: 1 oocyte per 10 μl drop) at 39 °C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 and 95% humidity. Development into blastocysts and hatched blastocysts was observed on day 8 (day 0 = the day of insemination).

Culture media

The basic culture medium was an SOF medium consisting of 117.7 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.33 mM sodium pyruvate and 0.75 mg/ml kanamycin sulphate. In addition, several modifications were made to this medium (see Table 1).

Experimental design

Immature oocytes were cultured in PVA-mSOFaa (SOF-IVM; Table 1) containing 0 (negative control), 1, 10 or 50 ng/ml EGF in both the group- and individual-IVP systems. Also, two further groups – TCM199 and mSOFaa supplemented with 10% (v/v) fetal calf serum (FCS) – were included as positive control. We examined the nuclear stages of bovine oocytes at 24 h after IVM, the fertilization of bovine oocytes at 18 h after IVF, embryonic development to the blastocyst stage 8 days after IVF, and the cell numbers of each blastocyst developed in the group- and individual-IVP systems.

Oocyte fixing and staining

To determine the nuclear stages following *in vitro* maturation, the proportion of oocytes at the second metaphase stage (MII) was calculated. To determine the proportion of fertilized oocytes, oocytes were examined for evidence of normal or polyspermic (more than two pronuclei and decondensed sperm heads) fertilization. Normal fertilization was defined as monospermic penetration with male and female pronuclei and a sperm tail; oocytes without a male pronucleus were not considered as normally fertilized. The cumulus-cell-free oocytes and putative zygotes were fixed in 25% (v/v) acetic acid in ethanol for 24 h, and in 1% (w/v) orcein in 45% (v/v) acetic acid solution.

Determination of cell number

Determination of the cell number of each blastocyst was performed with a modification of the method of Yoshizawa *et al.* (1998). Briefly, blastocysts were placed in 1% (w/v) sodium citrate solution for 20 min at room temperature and then transferred to a solution of sodium citrate solution containing solution I (methanol:acetic acid = 1:1) for 30–60 to lightly fix them. The fixed blastocysts were mounted on a glass slide, and fixed again with solution I and solution II (methanol:acetic acid = 3:1), respectively. Finally, glass slides were dried and staining with 10% (w/v) Giemsa solution for 15 min. After washing off the staining solution, the number of nuclei of each blastocyst was counted under a phase-contrast microscope (× 400).

https://doi.org/10.1017/S0967199404002710 Published online by Cambridge University Press

Table 2 Effect of adding EGF to IVM media on nuclear maturation of bovine oocytes matured in the group-IVP system

System	IVM treatment			No. of replicates	No. of examined oocytes	Metaphase II (% ± SEM)
	Protein	Medium	EGF (ng/ml)			
Group	FCS	TCM199	–	3	59	81.4 ± 2.3^{a}
		mSOFaa	–	3	58	77.6 ± 2.8^{ab}
	PVA	mSOFaa	0 (control)	3	59	62.7 ± 5.0^{c}
			1	3	65	80.0 ± 5.0^{ab}
			10	3	64	73.4 ± 6.5^{b}
			50	3	59	79.7 ± 2.4^{ab}

$^{a–c}$ Different superscripts in the same column indicate values are significantly different ($p < 0.05$).

Table 3 Effect of adding EGF to IVM media on nuclear maturation of bovine oocytes matured in the individual-IVP system

System	IVM treatment			No. of replicates	No. of examined oocytes	Metaphase II (% ± SEM)
	Protein	Medium	EGF (ng/ml)			
Individual	FCS	TCM199	–	3	40	75.0 ± 4.3^{ab}
		mSOFaa	–	3	42	69.0 ± 3.1^{ab}
	PVA	mSOFaa	0 (control)	3	42	57.1 ± 14.4^{b}
			1	3	42	76.2 ± 5.4^{a}
			10	3	46	69.6 ± 9.8^{ab}
			50	3	46	71.7 ± 2.9^{ab}

$^{a–b}$ Different superscripts in the same column indicate values are significantly different ($p < 0.05$).

Statistical analysis

The general linear models (GLM) procedure of a Statistical Analysis System (SAS) were used to examine for analysis of variance in each factorial study of experiments. Furthermore, Duncan's multiple range test of SAS was also carried out to obtain detailed information on significant differences among the treatments. The mean numbers of cells per blastocyst were compared by Student's *t*-test using SAS.

Results

No significant difference was observed in the proportions of matured oocytes between the two FCS-containing media (TCM199 and mSOFaa) in either culture systems (Tables 2, 3). In the group-IVP system (Table 2), EGF (1, 5 or 50 ng/ml) supplementations significantly ($p < 0.05$) increased the proportions of oocytes compared with the absence of EGF (control medium), and gave similar levels of maturation to the two media supplemented with FCS. In the individual-IVP system (Table 3), only the addition of 1 ng/ml EGF significantly ($p < 0.05$) increased the proportion of matured oocytes compared with the control medium.

No significant difference was observed in the proportions of penetrated oocytes between the two FCS-containing media in either culture system (Tables 4, 5). In the group-IVP system (Table 4), the proportions of penetrated oocytes in the control medium, or medium supplemented with 1 or 10 ng/ml EGF, were significantly ($p < 0.05$) lower than that in the FCS-containing TCM199. However, only the medium supplemented with 50 ng/ml EGF gave comparable results in the proportions of penetrated oocytes to the two FCS-containing media in the group-IVP system. In the individual-IVP system (Table 5), the control medium supplemented with EGF at all concentrations resulted in similar proportions of penetrated oocytes to that in the FCS-containing mSOFaa, and only 10 ng/ml EGF treatment showed significantly ($p < 0.05$) lower proportions of penetrated oocytes than that in the FCS-containing TCM199. All treatments gave similar proportions of normally fertilized oocytes and polyspermy rates in both culture systems.

No significant difference in the proportion of blastocysts developing was observed between the two FCS-containing media in either culture system (Tables 6, 7). In the group-IVP system (Table 6), there was no significant difference in the proportions of blastocysts developing amongst all treatments. On the other hand,

https://doi.org/10.1017/S0967199404002710 Published online by Cambridge University Press

Table 4 Effect of adding EGF to IVM media on *in vitro* fertilization of bovine oocytes inseminated in the group-IVP system

System	IVM treatment			No. of replicates	No. of inseminated embryos	Percentage of fertilized oocytes (mean ± SEM)		
	Protein	Medium	EGF (ng/ml)			Penetrated*	Normal fertilized†	Polyspermy†
Group	FCS	TCM199	–	3	44	88.6 ± 1.3^{a}	87.2 ± 5.4	12.8 ± 5.4
		mSOFaa	–	3	42	81.0 ± 5.8^{ab}	82.6 ± 5.6	17.6 ± 5.6
	PVA	mSOFaa	0 (control)	3	41	78.0 ± 3.5^{b}	87.5 ± 3.2	15.6 ± 3.2
			1	3	41	78.0 ± 5.0^{b}	90.6 ± 7.1	9.4 ± 7.1
			10	3	40	77.5 ± 5.0^{b}	90.3 ± 7.7	9.7 ± 7.7
			50	3	39	84.6 ± 5.9^{ab}	84.8 ± 10.4	15.2 ± 10.5

[a–b] Different superscripts in the same column indicate values are significantly different ($p < 0.05$).
* Proportions against the number of inseminated oocytes.
† Proportions against the number of penetrated oocytes.

Table 5 Effect of adding EGF to IVM media on *in vitro* fertilization of bovine oocytes inseminated in the individual-IVP system

System	IVM treatment			No. of replicates	No. of inseminated embryos	Percentage of fertilized oocytes (mean ± SEM)		
	Protein	Medium	EGF (ng/ml)			Penetrated*	Normal fertilized†	Polyspermy†
Individual	FCS	TCM199	–	4	42	85.7 ± 6.0^{a}	91.7 ± 9.9	9.1 ± 9.9
		mSOFaa	–	4	42	78.6 ± 3.5^{ab}	84.8 ± 10.5	17.9 ± 10.5
	PVA	mSOFaa	0 (control)	4	49	81.6 ± 6.5^{ab}	87.5 ± 6.9	14.3 ± 6.9
			1	4	52	76.9 ± 9.4^{ab}	90.0 ± 4.2	11.1 ± 4.2
			10	4	50	76.0 ± 9.2^{b}	89.5 ± 7.9	11.8 ± 7.9
			50	4	46	84.8 ± 9.0^{ab}	92.3 ± 5.6	8.3 ± 5.6

[a–b] Different superscripts in the same column indicate values are significantly different ($p < 0.05$).
* Proportions against the number of inseminated oocytes.
† Proportions against the number of penetrated oocytes.

Table 6 Effect of adding EGF to IVM media on development to the blastocyst stage of bovine oocytes and cell number of blastocysts in the group-IVP system

System	IVM treatment			No. of replicates	No. of cultured embryos	Percentage of embryos at blastocyst stage (mean ± SEM)	*n**	No. of cells per blastocyst (mean ± SEM)
	Protein	Medium	EGF (ng/ml)					
Group	FCS	TCM199	–	4	63	22.2 ± 4.7	30	106 ± 20
		mSOFaa	–	4	78	28.2 ± 10.6	35	96 ± 23
	PVA	mSOFaa	0 (control)	4	85	24.7 ± 6.7	34	97 ± 27
			1	4	63	30.2 ± 6.3	34	109 ± 22
			10	4	63	23.8 ± 10.4	29	118 ± 27
			50	4	70	27.1 ± 9.1	32	111 ± 17

* Number of blastocysts examined for cell number.

in the individual-IVP system (Table 7), the medium supplemented with 10 ng/ml EGF supported significantly ($p < 0.05$) more blastocyst development than those supplemented with EGF and a similar rate to the FCS-containing media. There was no significant difference among treatments in either culture system (Tables 6, 7) as regards cell numbers of blastocysts.

Discussion

TCM199 is a complex medium generally used as a basic medium for oocyte maturation and embryo culture. However, to optimize culture condition for oocytes or embryos, it is necessary to modify the composition of the medium to improve IVP results. Although, in the

https://doi.org/10.1017/S0967199404002710 Published online by Cambridge University Press

Table 7 Effect of adding EGF to IVM media on development to the blastocyst stage of bovine oocytes inseminated in the individual-IVP system

System	IVM treatment			No. of replicates	No. of cultured embryos	Percentage of embryos at blastocyst stage (mean ± SEM)	n^*	No. of cells per blastocyst (mean ± SEM)
	Protein	Medium	EGF (ng/ml)					
Individual	FCS	TCM199	–	5	77	19.5 ± 6.2^{ab}	35	112 ± 19
		mSOFaa	–	5	75	14.7 ± 5.9^{abc}	32	104 ± 27
	PVA	mSOFaa	0 (control)	5	81	6.2 ± 3.5^{c}	31	105 ± 20
			1	5	81	14.8 ± 7.4^{abc}	30	98 ± 24
			10	5	85	20.0 ± 5.2^{a}	36	112 ± 21
			50	5	70	11.4 ± 7.2^{bc}	28	117 ± 25

$^{a–b}$ Different superscripts in the same column indicate values are significantly different ($p < 0.05$).
* Number of blastocysts examined for cell number.

present study, mSOF (modified synthetic oviduct fluid) was used as a basic culture medium for IVM, IVF and IVC it may not be optimal for oocyte maturation and embryo development. However, since it is a completely defined and simple medium, it can easily be modified for the establishment of suitable culture conditions. The original SOF medium was developed from the biochemical analysis of ovine oviducal fluid (Tervit *et al.*, 1972). In the present study, several modifications were made from the original formulation according to other reports (Fukui, 1990; Holm *et al.*, 1999; Gandhi *et al.*, 2000). The present results show that the maturation, fertilization and developmental potentials of bovine oocytes cultured in mSOF were similar to those in TCM199-containing serum. This result suggests that the mSOF used in this study was an effective medium for bovine oocyte maturation *in vitro* as well as TCM199, and agrees with the findings of Lonergan *et al.* (1994) and Rose-Hellekant *et al.* (1998).

Culture systems have recently incorporated the use of a separate medium for each phase of *in vitro* embryo production. The effect of changing media in this way has improved IVP results (Gardner *et al.*, 1994; Pinyopummintr & Bavister, 1996). However, Fukui *et al.* (1996) and Gandhi *et al.* (2000) described that repeated medium changes might have a detrimental effect on the developmental competence of bovine embryos. Likewise, we consider that a single medium for an IVP system may simplify the preparation of media. The mSOF medium used in this study was always prepared from the same series of basic stock solutions. All components can be prepared separately in solution as a basic stock. Thus, stocks that have either short lives, such as glutamine and bicarbonate, or long lives, such as stable salts, are always fresh when used. In addition, time spent on medium preparation can be shortened.

It has been demonstrated that a variety of growth factors are synthesized in the ovary and have important roles in ovarian cell function, such as follicular development and proliferation of ovarian follicle cells (Dorrington *et al.*, 1987; Carson *et al.*, 1989; Hammond *et al.*, 1993). Among these growth factors, epidermal growth factor (EGF) specifically binds to its specific membrane receptor to activate the MAP-kinase cascade and MPF (Carpenter & Cohen, 1990), both of which are essential for maturation in mammalian oocytes (Fissore *et al.*, 1996; Inoue *et al.*, 1998; Anas *et al.*, 2000*c*). EGF concentration in bovine follicular fluid was not measured in the present study, but a concentration of 5–13 ng/ml has been reported in porcine follicular fluid (Hsu *et al.*, 1987). In the present study, we examined the effects of adding EGF to the IVM medium in both an individual- and group-IVP system using bovine oocytes. The results showed that the effect of EGF during *in vitro* oocyte maturation differed between the individual and group culture systems. In the group-IVP system, EGF at all concentrations during IVM improved the nuclear maturation of oocytes, but no beneficial effect was found in fertilization and developmental capacity. On the other hand, in the individual-IVP system, 10 ng/ml EGF during IVM improved developmental competence to the blastocyst stage. In fact, the present results indicate that adding EGF during IVM was more effective in promoting blastocyst development in the individual-IVP system than in the group-IVP system. However, it is difficult to declare the cause of the different effects of EGF as dependent on the culture system based on the present result. It has been reported that there is cooperative interaction among oocytes or embryos cultured in a group. For example, Thibodeaux *et al.* (1995) demonstrated a cooperative interaction in bovine embryos incubated in groups that is due, at least in part, to platelet-derived growth factor (PDGF). Furthermore, Singh *et al.* (1995) showed that EGF was synthesized by cumulus cells. Based on these observations, it is possible that the mutual stimulation originating from some factors such as insulin, PDGF and EGF, produced by oocyte and cumulus cells, led to the different effects of EGF in maturation medium between the group- and

https://doi.org/10.1017/S0967199404002710 Published online by Cambridge University Press

individual-IVP systems. Namely, in the individual-IVP system, because of the absence of such cooperative interaction, adding EGF to maturation medium may be more effective.

An individual-IVP system may allow the elucidation of the relationship between the follicular environment and the developmental competence of each oocyte. In addition this system, in the absence of the interaction observed in the group-IVP system, may provide more detailed information about oocyte maturation and embryonic development. However, in the present individual-IVP system using a chemically defined medium, blastocyst formation was low due to the storage of ovaries for 1 day at 20 °C. This long storage may have affected the initial quality of follicular oocytes, which in turn may have greatly affected subsequent embryonic development. The finding of this study was that bovine oocytes individually matured, fertilized and cultured in a chemically defined medium could develop to the blastocyst stage at a similar rate as oocytes in group culture containing serum, provided an appropriate dose of EGF (10 ng/ml) was employed. The addition of EGF to the maturation medium improved the blastocyst rate as previously reported by Fukui *et al.* (2000) and Mizushima & Fukui (2001). In the future, further studies such as improvement of the IVM system in terms of drop size, energy substrates and culture gas phase are needed to enhance the quality of blastocysts derived from the individual IVP method and the fertility after transfer of those blastocysts following freezing or vitrification.

In conclusion, bovine oocytes can successfully attain the blastocyst stage in an individual-culture system using a single, simple chemically defined medium (mSOF + PVA). This is the first report of such a finding in an individual-IVP system. Our results show that adding EGF during IVM increases both nuclear maturation and development to blastocysts in the defined individual-IVP system. Furthermore, this study suggests that adding EGF to the defined medium for bovine oocyte maturation can replace the addition of serum. The defined individual-IVP system used in the present study can provide more precise information on the competence of individual bovine oocytes aspirated from various antral follicles for development.

Acknowledgements

The authors wish to thank the Hokkaido Meat Board in Obihiro for supplying bovine ovaries. The authors also thank Dr J.G. Thompson for reading the manuscript and for his valuable criticism. The part of the study was financially supported by Grants-in-Aid Scientific Research (No. 13660276) from the Ministry of Education, Science, Sports and Culture of Japan.

References

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Rieke, A., Murphy, C.N., Prather, R.S. & Day, B.N. (2000). Development and viability of pig oocytes matured in a protein-free medium containing epidermal growth factor. *Theriogenology* **54**, 787–97.

Anas, M.K., Elmileik, A., Maeda, T. & Terada, T. (1998). Effect of epidermal growth factor, insulin like growth factor-I and insulin on meiotic maturation of bovine denuded oocytes. *J. Mamm. Ova. Res*. **15**, 179–84.

Anas, M.K., Shimada, M., Zeng, W. & Terada, T. (2000*a*). Meiotic maturation of bovine oocytes cultured *in vitro* with EGF and wortmannin. *J. Mamm. Ova. Res.* **17**, 103–8.

Anas, M.K., Shimada, M., Zeng, W. & Terada, T. (2000*b*). Wortmannin, a specific phophatidylinositol 3-kinase inhibitor, blocks *in vitro* fertilization and embryonic development of bovine oocytes matured *in vitro* with epidermal growth factor. *J. Mamm. Ova. Res*. **17**, 109–14.

Anas, M.K., Shojo, A., Shimada, M. & Terada, T. (2000*c*). Effects of wortmannin on the kinetics of GVBD and the activities of the maturation-promoting factor and mitogen-activated protein kinase during bovine oocyte maturation *in vitro*. *Theriogenology* **53**, 1797–806.

Araki, N., Sato, K., Hayashi, K. & Fukui, Y. (1997). Relationships among follicular fluid estradiol-17β concentration, morphology of cumulus–oocyte complex and developmental capacity of individually matured, fertilized and cultured bovine oocytes *in vitro*. *J. Reprod. Dev*. **44**, 359–65.

Berthelot, F. & Terqui, M. (1996). Effects of oxygen, CO_2/pH and medium on the *in vitro* development of individually cultured porcine one- and two-cell embryos. *Reprod. Nutr. Dev*. **36**, 241–51.

Carolan, C., Lonergan, P., Khatir, H. & Mermillod, P. (1996). *In vitro* production of bovine embryos using individual oocytes. *Mol. Reprod. Dev*. **45**, 145–50.

Carpenter, G. & Cohen, S. (1990). Epidermal growth factor. *J. Biol. Chem.* **265**, 7709–12.

Carson, R.S., Zhang, Z., Hutchinson, L.A., Herington, A.C. & Findlay, J.K. (1989). Growth factors in ovarian function. *J. Reprod. Fertil*. **85**, 735–46.

de Matos, D.G. & Furnus, C.C. (2000). The importance of having high glutathione (GSH) level after bovine *in vitro* maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. *Theriogenology* **53**, 761–71.

Dorrington, J.H., Bendell, J.J., Chuma, A. & Lobb, D.K. (1987). Actions of growth factors in the follicle. *J. Steroid Biochem*. **27**, 405–11.

Fissore, R.A., He, C.L. & Vande. Woude, G.F. (1996). Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes. *Biol. Reprod*. **55**, 1261–70.

Fukui, Y. (1990). Effect of follicle cells on the acrosome reaction, fertilization, and developmental competence of bovine oocytes matured in vitro. *Mol. Reprod. Dev*. **26**, 40–6.

Fukui, Y., Lee, E.S. & Araki, N. (1996). Effect of medium renewal during culture in two different culture systems on development to blastocysts from *in vitro* produced early bovine embryos. *J. Anim. Sci*. **74**, 2752–8.

Fukui, Y., Kikuchi, Y., Kondo, H. & Mizushima, S. (2000). Fertilizability and developmental capacity of individually cultured bovine oocytes. *Theriogenology* **53**, 1553–65.

https://doi.org/10.1017/S0967199404002710 Published online by Cambridge University Press

Gandhi, A.P., Lane, M., Gardner, D.K. & Krisher, R.L. (2000). A single medium supports development of bovine embryos throughout maturation, fertilization and culture. *Hum. Reprod*. **15**, 395–401.

Gardner, D.K., Lane, M., Spitzer, A. & Batt, P.A. (1994). Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol. Reprod*. **50**, 390–400.

Hagemann, L.J., Weilert, L.L., Beaumont, S.E. & Tervit, H.R. (1998). Development of bovine embryos in single *in vitro* production (sIVP) systems. *Mol. Reprod. Dev*. **51**,143–7.

Hagemann, L.J., Beaumont, S.E., Berg, M., Donnison, M.J., Ledgard, A., Peterson, A.J., Schurmann, A. & Tervit, H.R. (1999). Development during single IVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. *Mol. Reprod. Dev*. **53**, 451–8.

Hammond, J.M., Samaras, S.E., Grimes, R., Leighton, J., Barber, J., Canning, S.F. & Guthrie, H.D. (1993). The role of insulin-like growth factors and epidermal growth factor-related peptides in intraovarian regulation in the pig ovary. *J. Reprod. Fertil. Suppl*. **48**, 117–25.

Hendriksen, P.J.M., Bevers, M.M. & Dieleman, S.J. (1999). Single IVP using BRL cell co-culture and serum yields a lower blastocyst rate than group culure. *Theriogenology* **51**, 336.

Holm, P., Booth, P.J., Schmidt, M.H., Greve, T. & Callesen, H. (1999). High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology* **52**, 683–700.

Hsu, C.J., Holmes, S.D. & Hammond, J.M. (1987). Ovarian epidermal growth factor-like activity: concentrations in porcine follicular fluid during follicular enlargement. *Biochem. Biophys. Res. Commun*. **147**, 242–7.

Inoue, M., Naito, K., Nakayama, T. & Sato, E. (1998). Mitogen-activated protein kinase translocates into the germinal vesicle and induces germinal vesicle breakdown in porcine oocytes. *Biol. Reprod*. **58**, 130–6.

Kane, M.T. (1983). Variability in different lots of commercial bovine serum albumin affects cell multiplication and hatching of rabbit blastocysts in culture. *J. Reprod. Fertil*. **69**, 555–8.

Keefer, C.L., Stice, S.L., Paprocki, A.M. & Golueke, P. (1994). *In vitro* culture of bovine IVM-IVF embryos: cooperative interaction among embryos and the role of growth factors. *Theriogenology* **41**, 1323–31.

Keskintepe, L. & Brackett, B.G. (1996). *In vitro* developmental competence of *in vitro*-matured bovine oocytes fertilized and cultured in completely defined media. *Biol. Reprod.* **55**, 333–9.

Keskintepe, L., Burnley, C.A. & Brackett, B.G. (1995). Production of viable bovine blastocysts in defined *in vitro* conditions. *Biol. Reprod*. **52**, 1410–17.

Lonergan, P., Carolan, C. & Mermillod, P. (1994). Development of bovine embryos *in vitro* following oocyte maturation under defined conditions. *Reprod. Nutr. Dev*. **34**, 329–39.

Lonergan, P., Carolan, C., Van. Langendonckt, A., Donnay, I., Khatir, H. & Mermillod, P. (1996). Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. *Biol. Reprod*. **54**, 1420–9.

Mizushima, S. & Fukui, Y. (2001). Fertilizability and developmental capacity of bovine oocytes cultured individually in a chemically defined maturation medium. *Theriogenology* **55**, 1431–45.

O'Doherty, E.M., Wade, M.G., Hill, J.L. & Boland, M.P. (1997). Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology* **48**, 161–9.

Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L. & Critser, E.S. (1986). Bovine *in vitro* fertilization with frozen-thawed semen. *Theriogenology* **25**, 591–600.

Pinyopummintr, T. & Bavister, B.D. (1996). Energy substrate requirements for *in vitro* development of early cleavage-stage bovine embryos. *Mol. Reprod. Dev.* **44**, 193–9.

Rose-Hellekant, T.A., Libersky-Williamson, E.A. & Bavister, B.D. (1998). Energy substrates and amino acids provided during *in vitro* maturation of bovine oocytes alter acquisition of developmental competence. *Zygote* **6**, 285–94.

Singh, B., Rutledge, J.M. & Armstrong, D.T. (1995). Epidermal growth factor and its receptor gene expression and peptide localization in porcine ovarian follicles. *Mol. Reprod. Dev*. **40**, 391–9.

Tervit, H.R., Whittingham, D.G. & Rowson, L.E. (1972). Successful culture *in vitro* of sheep and cattle ova. *J. Reprod. Fertil*. **30**, 493–7.

Thibodeaux, J.K., Myers, M.W. & Hansel, W. (1995). The beneficial effects of incubating bovine embryos in groups are due to platelet-derived growth factor. *Theriogenology* **43**, 336.

Watson, A.J., De. Sousa, P., Caveney, A., Barcroft, L.C., Natale, D., Urquhart, J. & Westhusin, M.E. (2000). Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis. *Biol. Reprod*. **62**, 355–64.

Yoshizawa, M., Matsukawa, A., Matsumoto, K., Suzuki, K., Yasumatsu, K., Zhu S. & Muramatsu, S. (1998). Required concentration and time of vinblastine treatment for chromosome preparation in bovine blastocysts derived from *in vitro* fertilization. *J. Reprod. Dev*. **44**, 59–64.

https://doi.org/10.1017/S0967199404002710 Published online by Cambridge University Press