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Development to Hatching Blastocysts and Cell Allocation to the Inner Cell Mass and Trophectoderm of Pig *In Vitro* Embryos as Affected by Amino Acids and Serum

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아미노산과 혈청이 돼지 수정란의 내부세포괴와 영양배엽세포로의 발달과 부화에 미치는 영향

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체외성숙과 수정된 돼지 난자의 체외발달능이 체외배발생 배양액인 NCSU 배양액에 0.4% BSA, 10% 혈청 혹은 아미노산 (2% BME 아미노산 용액과 1% MEM 아미노산 용액)을 첨가함으로서 조사되었다. 본 실험에 공시된 난자는 체외수정 후 30 시간 (2-세포기) 혹은 48 시간 (2~4-세포기)에 회수하였다. 실험I에서 0.4% BSA가 첨가된 NCSU 배양액에서 2-세포기 난자들의 배양경과시간에 따른 발달능을 조사한 결과, 배양 후 72 시간 (체외수정 후 102 시간)에 상실배기와 배반포기 배가 나타났으며, 배양 후 120 시간째 (체외수정 후 150 시간)에도 팽창된 배반포기 배까지만 발달하였다. 실험II는 체외수정 후 48 시간의 분할된 (2~8-세포기) 난자들의 핵과 외관적 분할구와의 수적 차이를 조사한 결과, 2~4-세포기보다는 5-세포기 이상에서 핵과 분할구의 조화에 차이가 많았다. 실험III에서는 2~4-세포기 난자들을 배양후 5일째의 배반포들의 투명대의 두께, 난자 크기 그리고 inner cell mass (ICM)과 trophectoderm (TE)의 세포 배열을 조사한 결과, 난자의 크기가 커짐에 따라서 투명대가 얇아지고 전체 세포수가 증가하였지만, ICM의 비율은 차이가 없었다. 실험IV에서는 BSA, 혈청 혹은 아미노산이 첨가 혹은 무첨가된 배양액내에서 2~4-세포기 난자들의 배반포 후 부화능력을 조사한 결과, 모든 군에 있는 난자들은 팽창된 배반포기 배까지 발달할 수 있었던 반면, 난자의 부화는 아미노산 혹은 혈청이 포함된 배양액에서만 일어났다. 더우기 상실배기와 배반포기 시기에 혈청의 첨가는 부화 배반포기 배의 발달을 현저히 증가시켰다. 또한 아미노산과 혈청의 영향을 받은 팽창 배반포기 배는 얇은 투명대, 팽창된 난자의 크기 그리고 ICM과 전체 세포수의 증가를 보였다. 이상의 결과로 미루어 볼때, 배양액내에 대한 아미노산과 혈청의 첨가는 돼지 배반포기 배의 부화를 유도할 수 있다고 보며, 더우기 이들 요소들은 투명대의 두께, 난자의 크기 그리고 ICM과 전체 세포수에 영향을 미친다.

INTRODUCTION

Many researchers, in pig, have been investigated for *in vitro* culture system of immature oocytes (Mattioli *et al.*, 1989; Funahashi *et al.*, 1993; Yoshida *et al.*, 1993; Niwa, 1993; Nagai, 1994). However, development from immature oocytes to blastocysts *in vitro* a little limited in pig. Therefore, in pig, many reports have been more examined by *in vivo* than *in vitro* embryos compared with other species (Archibong *et al.*, 1989; Krisher *et al.*, 1989ab; Prather *et al.*, 1991; Rieger *et al.*, 1992).

Many factors (BSA, serum, amino acids, growth factors, etc.) for improvement of culture system have used as the additive in culture medium in pig as well as others (Stone *et al.*, 1984; Ding and Foxcroft, 1994; Harper and Brackett, 1993; Rosenkrans and First, 1994). Rosenkrans *et al.* (1989) reported that the blastocyst formation of bovine embryos was affected by the culture medium supplemented with amino acids. The presence of amino acids in the culture medium increases blastocyst formation, hatching, and cell number on the development of embryos (Stone *et al.*, 1984; Meyen *et al.*, 1989; Gardner and Lane, 1993; Rosenkrans and First, 1994). In pig, the development of zygotes in simple medium can develop to blastocyst, but the development of blastocysts ceases *in vitro* after blastocyst expansion (Wright, 1977; Menino and Wright, 1982; Petters *et al.*, 1990; Hagen *et al.*, 1991; Beckmann and Day, 1993). Hatching and trophoblast expansion after pig blastocyst formation were stimulated by including serum and amino acids in the culture medium (Robl and Davis, 1981; Stone *et al.*, 1984; Rosenkrans *et al.*, 1989; Pollard *et al.*, 1995). Pollard *et al.* (1995) suggested that the development of *in vivo* pig zygotes to blastocyst can be supported by simple culture media, but the development of morulae to hatching was superior to complex medium contained amino acids and serum. These results showed that development of embryos necessarily requires to nutrients and energy sources. These factors more affect at the continued development and hatching of late embryo stage after morula and blastocyst formation than cleavage of early embryo stage. Therefore, this report examined to effects of amino acids and serum on the blastocyst development as well as hatching after the blastocyst formation of pig IVM/IVF produced embryos. In addition, we tested whether addition of amino acids and serum can affect to the zona thickness, embryo size, and cell allocation to the ICM and trophectoderm after blastocyst formation.

MATERIALS AND METHODS

Recovery of immature oocytes and *in vitro* maturation (IVM)

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline (35 to 37℃) within 1 hr. The oocyte-cumulus complexes (OCCs) were recovered by aspiration from the follicles (2~6 mm in diameter) using a 10 ml disposable syringe fitted with an 18-gauge needle. The OCCs were washed three times with TL-HEPES (1 mg/ml BSA, low carbonate TALP; Parrish *et al.*, 1988) and the maturation medium, respectively. The OCCs (about 50 oocytes) were transferred into a 0.5 ml of maturation medium equilibrated for 2 hr in 5% CO_2 and 95% O_2 incubator under warm mineral oil in a four well culture dish. The maturation medium consisted of TCM-199 (with Earle's salts: Gibco, USA) supplemented with 25 mM $NaHCO_3$, 10% FBS (fetal bovine serum, Gibco), 0.2 mM pyruvate, 0.6 mM L-cysteine, 10 μg/ml p-FSH, and 25 μg/ml gentamycin. Culture was carried out at 39℃ in 5% CO_2 in air for 42~44 hr.

Sperm capacitation and *in vitro* fertilization (IVF)

Semen were collected from cauda of epididymis obtained from a local slaughterhouse. Semen (1 ml) were diluted into 5 ml Sp-TALP (Rosenkrans *et al.*, 1994), and the extender was removed by washing two times (1,000 rpm) for 5 min. Then the sperm pellet was resuspended with Sp-TALP, and motile sperm were collected by swimup separation after incubation for 10 min. Highly motile sperm were added into the fertilization medium containing about 50 oocytes/0.5 ml to make a final concentration of 2.5×10^5 sperm/0.5 ml. Incubation conditions for IVF were 5% CO_2 in air with saturated humidity at 39℃. The fertilization medium was consisted of fatty acid free bovine serum albumin (FAF-BSA: 6 mg/ml), 0.2 mM pyruvate, 2 μg/ml heparin, 18.2 μM penicillamine, 9.1 μM hypotaurine, 1.8 μM epinephrine, and 25 μg/ml gentamycin. The oocytes and sperm were incubated for 30 hr at 5% CO_2 in air with saturated humidity at 39℃.

Embryo culture

The fertilized embryos (at 30 hr after IVF) were washed in NCSU (0.4% BSA) medium before being placed in culture. Embryos (about 50 embryos) were transferred into a 0.5 ml of NCSU (0.4% BSA) medium under a layer of mineral oil equilibrated for 2 hr in 5% CO_2 and 95% O_2 incubator under warm mineral oil in a four well culture dish. At 18 hr after culture of embryos collected cleaved embryos. These embryos cultured under various culture media conditions. Media for experiments used that NCSU medium contained 0.4% BSA or 10% FBS, and NCSUaa medium modified by NCSU medium supplemented with 2% BME amino acids solution and 1% MEM non essential amino acid solution contained 0.4% BSA or 10% FBS. Culture was carried out at 39℃ in 5% CO_2 in air for 5 days.

Blastocyst classification

Blastocysts derived from culture of 2~4 cell embryos separated early, middle, expanded, hatching, and hatched blastocyst. From early to expanded blastocysts at 5 day after culture of 2~4 cell embryos were calculated zona thickness and embryo size by eyepiece micrometer (Olympus).

Examination of nuclear status

Nuclear status of 2-, 3-, 4-, 5-, 6- to 8-cells was identified at 48 hr after IVF by using bisbezimide (Hoechst 33342, Sigma). After the insemination, oocytes intended for direct fixation were stripped of cumulus cells and attached sperm by incubation with 1% hyaluronidase solution for 5 min. and pipetting, and zona pellucida of oocyte for more strip of attached sperm was removed by incubation with 0.05% protease for 1min. After the cumulus cells and zona pellucida were removed, the oocytes were fixed for a minimum of 10 min. in a buffered 2% formalin solution. The oocytes were then placed on a slide with a drop of mounting medium consisting of 1 : 1 glycerol: phosphate-buffered saline, containing 2.5 mg/ml sodium azide and 2.5 mg/ml Hoechst 33342 DNA label. A cover slip was placed on top of the embryos, and the edges were sealed with fingernail polish.

Differential labelling of ICM and TE nuclei

Total, TE and ICM nuclei of blastocysts were differentially labelled by using a method of Papaioannou and Ebert (1988) with some modifications. Briefly, TE nuclei are labelled first specifically with fluorochrome propidium iodide (PI, Sigma). This fluorochrome is excluded from viable ICM cell but labelled TE cells undergoing antibody-mediated complement lysis during immunosurgery. The whole embryo is rapidly fixed and both the TE and

ICM nuclei labelled with bisbenzimide. The protocol was as follows; embryo zona was removed in 0.5% pronase (Sigma) solution and allowed to recover for 10 min. in TL-Hepes. Embryos were incubated on ice for 10~15 min. in 15 mM TNBS (Sigma) containing 4 mg/ml PVP (Sigma) in TL-Hepes. After washing completely, embryos were incubated in 0.1 mg/ml anti-DNP-BSA (ICN Immunobiological.) in TL-Hepes for 10 min. at 37℃. After washing sufficiently in TL-Hepes, the embryos were incubated in 0.01 mg/ml PI and 10% (v/v) guinea pig complement (Sigma) in TL-Hepes for 15~30 min. at 37℃. After 15 min. Observed them until even lysis of the outer TE cells was seen and then transferred them into 0.05 mM bisbenzimide in absolute alcohol. After overnight storage at 4℃, the embryos were washed in absolute alcohol for at least 1 hr, and mounted in glycerol under a coverslip on a slide glass. Labelled nuclei were observed under ultra violet excitation filter incorporated fluorescent microscope and TE nuclei labelled with PI and bisbenzimide appeared pink or red, ICM nuclei labelled with bisbenzimide appeared blue or unlabelled.

Experimental design

In experiment I, the development of 2-cell embryos at 30 hr after IVF examined an interval of 24 hr for 5 days. In experiment II, the cleaved embryos (2- to 8-cells) at 48 hr after IVF compared number of nuclear status with morphological blastomeres. In experiment III, early, middle, expanded blastocysts produced at 5 day after culture of cleaved embryos (2- to 4-cell at 48 hr after IVF) compared with the zona thickness, embryos size, and the number of ICM and total cell. In experiment IV, cleaved embryos cultured in NCSU or NCSUaa supplemented with/without BSA or FBS. Also, the zona thickness, embryos size, and number of ICM and total cell blastocysts produced from these culture condition compared with those of NCSU supplemented with BSA (0.4%).

Statistical analysis

Chi-square test and Student t-test were used to ascertain statistical differences between treatments. A p value of less 0.0001, 0.005, 0.02, and 0.05 were considered statistically significant.

RESULTS

The development of 2-cell embryos (at 30 hr after IVF) examined for 5 days following time course (an interval of 24 hr) (Table 1).

Table 1. The development of 2-cell embryos at 30 hr after IVF according to time course

Stage	Time after 2-cell culture					
	0 hr	24 hr	48 hr	72 hr	96 hr	120 hr
2-cell	51 (100.0)	8 (15.7)	3 (5.9)	2 (3.9)	2 (3.9)	2 (3.9)
3-cell		15 (29.4)	5 (9.8)	2 (3.9)		
4-cell		21 (41.2)	27 (52.9)	15 (29.4)	4 (7.8)	1 (2.0)
5-cell		5 (9.8)	10 (19.6)	13 (25.5)	3 (5.9)	3 (5.9)
≥ 6-cell		2 (3.9)	6 (11.8)	8 (15.7)	3 (5.9)	4 (7.8)
Morula				11 (21.6)	29 (56.9)	19 (37.3)
Blastocyst					10 (19.6)	22 (43.1)
Early					9	2
Middle					1	9
Expanded						11

Table 2. Nuclei number of cleaved embryos at 48 hr after IVF

Stage	2-cell	3-cell	4-cell	5-cell	6~8-cell
No.	31	31	34	35	35
Mean±SD	2.0±0.00	2.4±0.66	3.5±0.75	3.6±1.21	4.1±2.14

Table 3. Zona thickness and embryo size (mean ± SEM) of various blastocysts at 7 day after IVF

Stage	No.	Zona thickness (Range)	Embryo size (Range)
Early	14	16.3 ± 0.6[a] (12.0 ~ 19.2)	155.8 ± 1.9[a] (144.0 ~ 170.4)
Middle	16	11.1 ± 0.7[b] (7.2 ~ 16.8)	174.9 ± 2.6[b] (156.0 ~ 189.6)
Expanded	18	5.2 ± 0.6[c] (2.4 ~ 9.6)	235.7 ± 5.0[c] (201.6 ~ 273.6)

[a-c] Different superscripts within columns were significantly different ($p<0.0001$).

Table 4. Number of ICM and total cells (mean ± SEM) in blastocysts at 7 day after IVF

Stage	No.	Total cells	ICM	
		No. of cells (Range)	No. of cells (Range)	Proportion (%)
Early	26	16.8 ± 1.5[a] (8 ~ 31)	3.6 ± 0.4[a] (1 ~ 8)	21.2 ± 1.8
Middle	32	32.3 ± 2.0[b] (16 ~ 58)	6.2 ± 0.7[b] (2 ~ 20)	20.8 ± 2.2
Expanded	33	59.1 ± 3.3[c] (31 ~ 109)	11.8 ± 0.9[c] (3 ~ 23)	20.6 ± 1.4

[a-c] Different superscripts within columns were significantly different ($p<0.05$).

The development of morula and blastocyst showed at 72 hr (at 102 hr after IVF) and 96 hr (at 126 hr after IVF) after the collection of 2-cell, respectively. The blastocyst at 120 hr (at 150 hr after IVF) developed to expanded blastocyst. Table 2 shows from 2- to 8-cell embryos (at 48 hr after IVF) when compared the number of morphological blastomeres with nuclei status. This experiment was to examine whether cleaved embryos develop normal or abnormal embryos. The harmony of the number between nuclei and blastomeres more differed from >4-cell than 2- to 4-cell embryos. From 2- to 4-cell embryos used for this experiment cultured during 5 days, and blastocysts produced from these embryos were separated early, middle, and expanded blastocyst by zona thickness and embryo size (Table 3). Zona thickness according to the expanding embryo size of blastocysts gradually thinned. Morever, the number of total cell of blastocysts according to the thinning zona thickness and expanding embryo size was increased, but the proportion of ICM was not different (Table 4). However, embryos under this culture condition can develop to expanded blastocyst, but the development as hatch after expanded blastocyst of these embryos is very difficult.

Therefore, the development as hatch blastocyst after blastocyst formation led to the culture media containing amino acids and serum used 2- to 4-cell embryos (Table 5). In all groups culture embyos can develop to expanded blastocyst, but the rates of blastocysts more enhance to the groups in the presence than absence BSA or serum, and the distribution of blastocysts produced in groups contained serum at initiation of 2~4-cell embryos more have early and middle than expanded blastocysts. However, hatching of blastocysts can be occurred by groups in the presence of amino acids or serum. Especially, the hatching rate of blastocysts was enhanced by morulae and early blastocysts developed from 2- to 4-

Table 5. Effects of amino acids and FBS on the development of 2- to 4-cell embryos at 48 hr after IVF

Treatment	No.	*Transfer	Blastocyst (5 day)				Hatch (7 day)		
			Total	I	II	III	Total	IV	V
NCSU	182	−	23 (12.6)[a]	11	1	11	0 (0.0)[a]	0	0
① NCSU	182	−	70 (38.5)[b]	11	14	45	0 (0.0)[a]	0	0
② NCSU	182	−	68 (37.4)[b]	35	13	20	15 (8.2)[b]	4	11
① NCSU	182	+	91 (50.0)[c]	22	24	45	32 (17.6)[c]	7	25
NCSUaa	182	−	24 (13.2)[a]	11	4	9	0 (0.0)[a]	0	0
① NCSUaa	182	−	68 (37.4)[b]	21	16	31	11 (6.0)[b]	5	6
② NCSUaa	182	−	80 (44.0)[bc]	34	23	23	15 (8.2)[b]	6	9
① NCSUaa	182	+	96 (52.7)[c]	23	19	54	34 (18.7)[c]	6	28

I: Early, II: Middle, III: ≥Expanded, IV: Hatching, V: Hatched.
① Supplemented with 0.4% BSA.
② Supplemented with 10% FBS.
* Exchange of culture media from 0.4% BSA to 10% FBS at 3 day after culture of embryos.
[a-c] Different superscripts within columns were significantly different ($p<0.02$).

Table 6. Zona thickness and embryo size (mean ± SEM) of expanded blastocysts produced from 2-4 cell embryos by various treatment at 48 hr after IVF

Treatment	*Transfer	No.	Zona thickness (Range)	Embryo size (Range)
NCSU	−	26	6.97 ± 0.56[a] (2.4 ~ 12)	210.7 ± 4.0[ab] (180 ~ 244.8)
① NCSU	−	21	6.74 ± 0.70[a] (2.4 ~ 12)	226.0 ± 5.4[bc] (198.4 ~ 291.2)
② NCSU	−	17	7.13 ± 0.49[a] (2.4 ~ 9.6)	202.9 ± 4.7[a] (182.4 ~ 264)
② NCSU	+	29	4.92 ± 0.44[bc] (2.4 ~ 12)	243.2 ± 3.4[d] (204 ~ 271.2)
NCSUaa	−	16	6.97 ± 0.73[a] (2.4 ~ 9.6)	211.9 ± 6.7[ab] (180 ~ 264)
① NCSUaa	−	40	4.86 ± 0.34[c] (2.4 ~ 9.6)	231.5 ± 3.6[cd] (196.8 ~ 288)
② NCSUaa	−	17	6.07 ± 0.40[ab] (3.6 ~ 9.6)	200.8 ± 4.6[a] (180 ~ 252)
② NCSUaa	+	43	5.16 ± 0.27[bc] (2.4 ~ 9.6)	238.4 ± 2.9[d] (201.6 ~ 276)

① Supplemented with 0.4% BSA.
② Supplemented with 10% FBS.
* Exchange of culture media from 0.4% BSA to 10% FBS at 3 day after culture of embryos.
[a-d] Different superscripts within columns were significantly different ($p<0.05$).

cell embryos transferred into the culture medium contained serum. Also, expanded blastocysts at 5 day after culture from these all groups examined to the zona thickness and embryo size (Table 6). The expanded blastocysts of groups transferred into the culture medium contained serum at 3 day after culture of 2~4-cell embryos and of group cultured in the culture medium (contained BSA) supplemented with amino acids showed the more thinning zona thickness and expanding embryo size than other groups, but expanded blastocysts of groups cultured in the medium contained serum from 2~4-cell embryos showed the less thinning zona thickness and expanding embryo size than those of the other groups. The number of ICM and total cells of expanded blastocysts of these all groups showed in Table 7. The number of total cells on the groups cultured in the culture medium contained amino acids (except group cultured in the medium contained serum from 2~4-cell embryos) and group transferred into the culture medium contained serum at 3 day after culture of 2~4-cell embryos more increased than those of the other groups. The proportion of ICM on the groups cultured in

Table 7. Number of ICM and total cells (mean ± SEM) in expanded blastocysts produced by treatments of amino acids or serum during 5 days on 2-4 cell embryos at 48 hr after IVF

Treatment	*Transfer	No.	Total cell	ICM	
			No. of cell (Range)	No. of cell (Range)	Proportion (%)
NCSU	−	25	52.4 ± 4.1^{a} (22 ~ 86)	8.6 ± 1.3^{a} (2 ~ 29)	16.8 ± 2.2^{ab}
① NCSU	−	23	58.4 ± 4.5^{ab} (25 ~ 109)	10.4 ± 1.1^{a} (3 ~ 19)	18.4 ± 1.6^{ab}
② NCSU	−	13	50.0 ± 6.0^{a} (26 ~ 101)	17.0 ± 1.5^{bc} (9 ~ 25)	36.0 ± 2.5^{c}
① NCSU	+	49	72.7 ± 3.7^{c} (33 ~ 165)	14.2 ± 1.0^{b} (4 ~ 27)	19.9 ± 1.0^{b}
NCSUaa	−	16	70.3 ± 6.6^{bc} (43 ~ 116)	8.6 ± 1.6^{a} (2 ~ 18)	13.4 ± 1.9^{a}
① NCSUaa	−	30	85.1 ± 6.3^{c} (23 ~ 137)	15.6 ± 2.2^{bc} (4 ~ 53)	19.0 ± 2.1^{b}
② NCSUaa	−	14	49.3 ± 4.8^{a} (30 ~ 93)	15.7 ± 1.6^{bc} (6 ~ 24)	32.3 ± 2.4^{cd}
① NCSUaa	+	30	74.3 ± 6.0^{c} (35 ~ 144)	20.3 ± 2.2^{c} (5 ~ 66)	27.4 ± 1.6^{d}

① Supplemented with 0.4% BSA.
② Supplemented with 10% FBS.
* Exchange of culture media from 0.4% BSA to 10% FBS at 3 day after culture of embryos.
[a-d] Different superscripts within columns were significantly different ($p<0.05$).

the medium contained serum from 2~4-cell embryos and group transferred into the culture medium (supplemented with amino acids) contained serum at 3 day after culture of 2~4-cell embryos more increased than those of the other groups.

DISCUSSION

The *in vitro* development of pig embryos has been shown that the development of pig embryos to blastocyst can be developed in simple culture media, but the development of pig embryos in complex media cannot develop to blastocyst because embryos become cease at early cleavage (4-cell) stage (Wright, 1977; Menino and Wright, 1982; Petters *et al.*, 1990; Hagen *et al.*, 1991; Beckmann and Day, 1993). Although pig embryos can develop to blastocyst, continued development and hatching of blastocysts *in vitro* are significantly less than the development *in vivo* (Pope and Day, 1972; Davis, 1985). However, this report shows that the addition of amino acids and serum to NCSU medium increased the development rates of blastocyst as well as hatching of pig IVM/IVF produced embryos.

In pig, morulae and early blastocysts formation usually appeared at day 5 after onset of oestrus, and escape from the zona pellucida of blastocysts occurred on the day 7 (Papaioannou and Ebert, 1988). The blastocoel formation by our data (Table 1) observed at day 5 after IVF, and expanding blastocyst and hatching of blastocyst occurred on the day 7 after IVF (Table 5). These results are very similar to those of previous researcher (Papaioannou and Ebert, 1988). The occurrence of 2-cell embryo appears after about 30 hr after IVF. The various cleaved embryos can observe after day 2 (after IVF), but a part of these embryos observed much difference from the number between morphological blastomeres and nuclei (Table 2). The average of number between blastomeres and nuclei of >4-cell embryos appeared to the difference of over one. A part of blastomere of these embryos suggest to the fragmented blastomere from cytoplasm, and the ability of development of these embryos (abnormal) was less than that of normal (2- to 4-cell) embryos (Data not shown). Morphological criterion of blastocysts derived these (2~4-cell) embryos was affected to the zona thickness according to the embryo size (Table 3). Total cell of blastocysts derived these embryos gradually increased the number according to the

thinning zona thickness and expanding embryo size, but the proportion of ICM was not different among blastocysts (Table 4).

On the other hand, continued development after blastocyst formation of normal embryos can develop as hatching in the presence of amino acids or serum in the culture medium, but these embryos cannot develop after expanded blastocyst in the absence of amino acids or serum in the culture medium (Table 5). Amino acids present high levels in oviduct and uterine fluids (Fahning *et al.*, 1967; Stanke *et al.*, 1974; Miller and Schultz, 1987; Gardner and Leese, 1990). Amino acids on many mammalian species can stimulate the development of embryos (Stone *et al.*, 1984; Meyen *et al.*, 1989; Gardner and Lane, 1993; Rosenkrans and First, 1994). The development of bovine embryos *in vitro* was improved by the addition of free amino acids to a simple medium (Rosenkrans and First, 1994). Stone *et al.* (1984) reported that MEM containing 13 amino acids supplemented with serum increased hatching and trophoblast expansion after blastocyst formation. In case of morulae and early blastocysts stage (day 5 after IVF) cultured in serum-free media (supplemented with/without amino acids), when transferred into the culture media (supplemented with/without amino acids) contained serum, hatching from expanded blastocysts of these embryos very significant increased to the comparison with other culture conditions (Table 5). The effect of serum on the development of embryos to hatching reported by many researchers (Robl and Davis, 1981; Stone *et al.*, 1984; Pollard *et al.*, 1995). Pollard *et al.* (1995) reported that zygotes cultured in complex medium compared with simple media cannot develop to blastocyst, but the development of morulae cultured in simple media contained serum and complex medium after culture in serum-free simple media was significantly increased to hatching after blastocyst formation compared with blastocysts cultured in serum-free simple media.

Morever, zona thickness and embryo size of expanded blastocyst showed the more thinning zona thickness and expanding embryo size in groups transferred into the culture medium contained serum at morula and early blastocyst stage and group (contained BSA) supplemented with amino acids than others (Table 6). The developments as hatching of blastocysts in these groups (thinning zona thickness and expanding embryo size) more effect than those of the other groups. However, expanded blastocysts of the groups (cultured in the culture medium contained serum from 2~4-cell embryos) showed the less thinning zona thickness and expanding embryo size than expanded blastocysts of the others, but some blastocysts of these groups can develop as hatching of blastocysts. Perhaps, the hatching of blastocyst, as well as zona thickness and embryo size, is affected by amino acids and serum containing many unknown factors.

Meyen *et al.* (1989) suggested that serum had significantly increase of nuclei number on the development of pig blastocysts. In cattle, the total number of cell in blastocysts was increased by amino acids in medium (Takahashi and First, 1992). Our experiment identified that the number of total cell of expanded blastocysts was increased when cultured in culture media supplemented with serum from morula and early blastocyst stage and amino acids from 2~4-cell embryos, and the proportion of ICM of expanded blastocysts enhanced under culture conditions cultured in culture media supplemented with serum from 2~4-cell embryos and in culture medium (contained amino acids) with serum from morula and early blastocyst stage (Table 7). In addition, expanded blastocysts cultured in culture media supplemented with medium (contained amino acids) with serum from morula and early blastocyst stage enhanced the proportion of ICM as well as the number of total cell. These results sug-

gest that zona thickness, embryo size, the number of total cell, and the proportion of ICM as well as hatching devlopment of blastocyst were affected by amino acids and serum. Specifically, morphology and energy requirements of embryos change at morula stage, and amino acids and serum efficiently use at this time. Therefore, this time may be require various energy sources and nutrients.

In conclusion, hatching after blastocyst formation of *in vitro* embryos can be improved by the addition of amino acids or serum in the culture medium. Especially, the addition of serum in morula stage can increase to the development of embryos to hatching. Therefore, this result suggests that amino acids and serum in the culture medium can support to the continued development and hatching of pig blastocysts.

CONCLUSION

In vitro development potential of pig IVM/IVF produced embryos was investigated in culture medium (NCSU) supplemented with BSA (0.4%), serum (10%), or amino acids (2% BME amino acids solution and 1% MEM non essetial amino acid solution). Embryos were collected at 30 hr (2-cell) or 48 hr (2- to 4-cells) after IVF. In experiment I, when the development ability of 2-cell embryos in the culture medium (NCSU + 0.4% BSA) following time course was examined, morulae and early blastocysts revealed at 72 hr (102 hr after IVF) after culture, and expanded blastocysts showed at 120 hr (150 hr after IVF). In experiment II, the cleaved (2- to 8-cell) embryos at 48 hr after IVF compared number of nuclei status with morphological blastomeres. The harmony of the number between nuclei and blastomeres more differed from over 4-cell than 2- to 4-cell embryos. In experiment III, zona thickness, embryo size, and cell allocation to the inner cell mass (ICM) and trophectoderm (TE) of blastocysts at 5 day after culture of 2-4 cell embryos were examined. According to expanding embryo size, zona thickness thinned and the number of total cell increased, but not different the proportion of ICM. In experiment IV, when the ability of hatching after blastocyst formation of 2- to 4-cell embryos in the culture medium supplemented with/without BSA, serum, or amino acids was examined, embryos in all groups can develop to expanded blastocyst, but hatching of embryos can be developed by the culture medium containing amino acids or serum. Morever, the addition of serum at morula and early blastocyst stages was significantly enhanced the hatching rate of blastocysts. Also, expanded blastocysts cultured in amino acids and serum showed the thinning zona thickness, the expanding embryo size, and the increasing number of ICM and total cell. Therefore, this result suggests that amino acids and serum in the culture medium can support to the continued development and hatching of pig blastocysts. In addition, these factors affect to the zona thickness, embryo size, the number of total cell, and the proportion of ICM.

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