FULL PAPER *Theriogenology*

Improved Development of ICR Mouse 2-Cell Embryos by the Addition of Amino Acids to a Serum-, Phosphate- and Glucose-Free Medium

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ABSTRACT. This study was conducted to evaluate how exogenous amino acids could affect preimplantation development of ICR mouse embryos. Two-cell embryos collected from naturally mated mice were cultured in amino acid-, glucose- and phosphate-free preimplantation (P)-1 medium. In Experiments 1, 19 amino acids (aa; 1% and 0.5% of MEM essential and nonessential amino acid solutions, respectively) were added to P-1 medium supplemented with either fatty acid-free bovine serum albumin (BSA; 3 mg/mL) or human follicular fluid (hFF; 10%). Regardless of BSA or hFF addition, embryo development to the morula (84 to 86% vs. 97 to 100%) and the blastocyst (54% vs. 93 to 94%) stages was significantly ($P<0.05$) enhanced by the addition of aa compared with no addition. In Experiment 2, the cell number of blastomeres and inner cell mass (ICM) cells in blastocysts and the ratio of ICM cell to trophectodermal cell (TE) were evaluated after aa addition. In both BSA- and hFF-containing P-1 medium, a significant increase in total blastomere number were found after aa addition (47 to 52 vs. 62 to 63 cells) compared with no addition. However, the ICM/TE ratio was not significantly affected by aa supplementation in both media, while ICM cell number was greatly increased after aa addition in hFF-containing medium (12 vs. 17 cells). When blastocysts were further cultured up to 162 hr post-hCG injection, development to the hatched blastocyst stage was significantly promoted by aa addition (0% vs. 11 to 20%) in both BSA- and hFF-containing media. In conclusion, aa significantly promote the preimplantation development to the hatched blastocyst stage and such effect mainly exerted on supporting blastomere proliferation.

KEY WORDS: amino acid, blastocyst, embryo culture, mouse, preimplantation development.

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The development of an effective culture medium for supporting preimplantation development of embryos in various animal species contributes to establishing innovative technologies in the fields of animal biotechnology and medical science. In the case of mouse embryo culture, strain specificity greatly influences the efficiency of culture medium and, to date, no conventional culture media have effectively supported "block" strain ICR mouse embryos that frequently used in various experimental model systems. To establish an effective culture system for this strain, specific substances, which were proven to have embryotropic effect, should be added to culture medium and putative embryotoxic substrates must be deleted from the culture system.

It has been reported that glucose and/or phosphate significantly inhibits preimplantation development in the mouse [21], cattle [13], pig [20], rat [18] and sheep [25] species, while glucose stimulates later preimplantation development after embryonic genome activation [13]. Accordingly, the use of glucose- and phosphate-free culture medium may be one of choices to promote *in vitro* development of ICR mouse embryos. On the other hand, the use of amino acids as a medium supplement may be beneficial for effectively supporting embryo development, since there were numerous reports on the stimulatory effect of amino acids on preimplantation development in various species [8, 9, 11, 13, 15, 16, 23].

Consequently, this study was designed to examine how amino acids affect the preimplantation development of ICR mouse embryos cultured in glucose- and phosphate-free medium. Two-cell embryos derived from *in vivo* and preimplantation-1 (P-1) medium were used in this study. In addition to routine examination of embryo development, the cell number of inner cell mass (ICM) and trophectoderm in blastocysts was counted to evaluate both proliferation and differentiation during *in vitro* preimplantation development. Since our laboratory has been used BSA or human follicular fluid (hFF) as a medium supplement, the effect of amino acids was examined in the presence of these macromolecules in P-1 medium.

MATERIALS AND METHODS

Collection of embryos: Four-weeks old female ICR mice were maintained under the controlled lighting conditions (14L: 10D) and superovulated by the injection of 5 I.U. PMSG (Folligon; Intervet Co., The Netherlands). At 48 hr after PMSG injection, 5 I.U. hCG (Chorulon; Intervet Co., The Netherlands) was administered and mating was concomitantly initiated at the time of hCG injection. The pres-

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ence of vaginal plug was examined at 16 hr post-hCG injection, and embryos developed to the 2-cell stages were collected from the oviduct of mated mice at 42 hr post-hCG injection. After washing several times in culture medium, embryos were provided for each experiment.

Medium: The basic medium used for the culture of mouse embryos was P-1 medium (Irvine Scientific Co., San Diego, CA). This medium consists of 101.6 mM NaCl, 4.69 mM KCl, 2.04 mM $CaCl_2 \cdot 2H_2O$, 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.33 mM sodium pyruvate, 21.4 mM sodium lactate, 25 mM $NaHCO_3$ and 0.15 mg/mL (w/v) sodium citrate, 10 μg/mL gentamycin and 5 mg/mL phenol red. This medium does not contain amino acids, glucose, inorganic phosphate and protein. According to the experimental design, hFF (10%, v/v), BSA (cat. no. A-4161, 3 mg/mL, Sigma Co., St. Louis, MO) and/or basal medium Eagle (BME) nonessential (0.5%, v/v) and essential (1%, v/v) amino acids solutions (aa; Gibco BRL, Grand Island, NY) were added to P-1 medium. The Institutional Review Board approved the collection of hFF in January 1999 and all information was given to consent patients. hFF was collected from stimulated women those who yielded large number of mature oocytes with excellent quality and with more than 50% of fertilization rate after IVF. After centrifuged twice at 150-G and inactivated at 56°C for 30 min, collected supernatant was filtered through 0.22 μm and stored at –70°C until use. The osmolarity and pH of the medium after the addition of tested substances were within the range of 295 to 305 mOsm and 7.3 to 7.4, respectively.

Culture of embryos and assessment of preimplantation development: A group of 10 to 14 embryos were cultured in 5 μL droplet of P-1 medium supplemented with different substances at 37°C, 5% CO_2 in humidified air atmosphere. Medium was equilibrated in such atmosphere at least for 3 hr prior to culture and the droplets of equilibrated medium were covered with warm mineral oil (BDH Co., Poole, Dorset, England). No medium change was made in the whole experiments, for avoiding the dilution of embryotropic substances that might be secreted from embryos themselves. The number of embryos developed to the 4-cell, 8-cell, morula, blastocyst, expanded blastocyst and hatched blastocyst stages were monitored under an inverted microscope (Eclipse TE300, Nikon, Tokyo, Japan) at 66, 90, 114, 138, 162, and 162 hr post-hCG injection, respectively.

Assessment of the proliferation and the differentiation of blastocysts: To evaluate the proliferation and differentiation of embryos, the cell numbers of blastocysts, and trophectoderm and ICM in the blastocyst were counted by the method of Hardy *et al.* [12] with a slight modification. The zona pellucida of blastocysts obtained at 138 hr post-hCG injection was removed by 0.5% (v/v) protease solution (cat. no. P-8811, Sigma) and the blastocysts were placed in 15 mM trinitrobezene sulfonic acid (cat. no. P-2297, Sigma) for 15 min at 4°C. Blastocysts were then incubated for 10 min in Tyrode's lactate solution supplemented with 25 mM Hepes and 0.1 mg/mL anti-dinitrophenol-BSA (cat no. 61-007-1, ICN, Irvine, CA, U.S.A.) at 39°C. Subsequently, they were treated with 0.01 mg/mL propidium iodide (cat. no. P-4170, Sigma) and incubated with 15% (v/v) guinea pig complement (cat. no. S-1639, Sigma) for 20 to 30 min at 39°C. The blastocysts were then placed in absolute ethanol solution supplemented with 0.05 mM fluorochrome bisbenzimide (cat. no. B-2261, Sigma) overnight at 4°C. After washing in absolute ethanol, the stained blastocysts were mounted on a glass slide and examined cell numbers under an inverted microscope with epifluorescent apparatus (HB-10104AF, Nikon, Tokyo, Japan).

Experimental design and statistical analysis: Concentrations of all tested substances were determined from the results of the preliminary experiment (data not shown). In Experiment 1, the effects of aa on the preimplantation development of mouse 2-cell embryos were evaluated. Two-cell embryos were randomly allocated into four groups and subsequently cultured up to 138 hr post-hCG injection in P-1 medium supplemented as follows; 1) BSA, 2) BSA + aa, 3) hFF and 4) hFF + aa. In Experiment 2, blastocyst were obtained following culture in BSA- or hFF-containing P-1 medium supplemented with or without aa and the number of total blastomeres and ICM cells and the ratio of ICM cell to trophectodermal cells at 138 hr after hCG injection was evaluated. In Experiment 3, 2-cell embryos were cultured in P-1 medium supplemented with BSA or hFF and with or without aa up to 162 hr post-hCG injection and the development of blastocysts to the hatched blastocyst stages was monitored.

Each experiment was replicated five times. Embryos developed to the 4-cell, 8-cell, morula, blastocyst, expanded blastocyst and hatched blastocyst stages were individually scored as a '1' (developed). Embryos that did not develop to the appropriate stages were scored as a '0' (not developed). The scores in each stage of development, mean number of total blastomeres and ICM cells in blastocysts, and the ICM cell to trophectodermal (TE) cell ratio were subjected to analysis of variance using the general linear model (PROC-GLM) in SAS program [1]. When the significance of the main effects was detected in each experimental parameter, the treatment effects were compared by the least square method.

RESULTS

Experiment 1: A total of 256 two-cell embryos were provided for this experiment. As shown in Table 1, a significant model effect of aa addition was found in the development to the 8-cell ($P=0.0097$), morula ($P=0.0097$) and blastocyst ($P=0.0001$) stages. Regardless of the addition of BSA or hFF in P-1 medium, more embryos developed to the blastocyst (93 to 94% vs. 54%, $P<0.0001$) stage after the addition of aa than after no addition. Similar trend was found in the development to the 8-cell and morula stages (97 to 100% in aa vs. 84 to 86% in no addition, $P<0.0456$) in both BSA- and hFF-containing media.

Experiment 2: A total of 124 two-cell embryos were provided for this study and 93 blastocysts were counted their

Table 1. Effects of the addition of amino acids (aa) to preimplantation (P)-1 medium supplemented with either bovine serum albumin (BSA)- or human follicular fluid (hFF) on development of ICR mouse 2-cell embryos to the blastocyst stage

P-1 medium supplemented with	With (+) or without (-) amino acids	No. of embryos cultured	No. (%)[a] of 2-cell embryos developed to			
			4-cell [66][b]	8-cell [90][b]	Morula [114][b]	Blastocyst [138][b]
BSA	–	63	63 (100)	53 (84)[c]	53 (84)[c]	34 (54)[c]
	+	68	68 (100)	66 (97)[d]	66 (97)[d]	64 (94)[d]
HFF	–	65	65 (100)	56 (86)[cd]	56 (86)[c,d]	35 (54)[c]
	+	60	60 (100)	60 (100)[e]	60 (100)[e]	56 (93)[d]

P value for analyzing the model effect was 0, 0.0097, 0.0097 and 0.0001 in 4-cell, 8-cell, morula and blastocyst, respectively.
a) Percentage of the number of 2-cell embryos cultured.
b) Hours post-hCG injection.
c-e) Different superscripts within each column are significantly different, P<0.05.

Table 2. Effects of addition of amino acids (aa) to preimplantation (P)-1 medium supplemented with bovine serum albumin (BSA) or human follicular fluids (hFF) or on cell numbers of inner cell mass (ICM) and trophectodermal (TE) cells in blastocysts[a] derived from ICR mouse 2-cell embryos

P-1 medium supplemented with	With (+) or without (-) aa	No. of embryo cultured	No. of blastocysts		Cell numbers[c] of (Mean ± SD)		Ratio of ICM /TE cells
			Obtained (%)[a]	Successfully labeled (%)[b]	Total blastomeres	Inner cell mass (ICM) cells	
BSA	–	29	17 (59)	15 (88)	52 ± 11[d]	14 ± 4[d]	0.34
	+	32	30 (94)	25 (83)	62 ± 18[e]	16 ± 5[d,e]	0.34
HFF	–	31	18 (58)	15 (83)	47 ± 14[d]	12 ± 9[d]	0.32
	+	32	28 (88)	22 (79)	63 ± 17[e]	17 ± 5[e]	0.36

P value for analyzing the model effect was 0.003, 0.0036 and 0.1046 in cell numbers of blastocysts and inner cell mass cells, and ICM cell to trophoblast ratio, respectively.
a) Percentage of the number of embryos cultured.
b) Percentage of the number of blastocysts obtained.
c) Cell numbers were counted on 162 hr post-mating.
d,e) Different superscripts within each column are significantly different, P<0.05.

cell numbers (Table 2). Of those, 77 were successfully labeled and 79 to 88% of blastocysts in each group was examined. A significant treatment effect was observed in the cell numbers of blastocysts (P=0.0036) and ICM cells (P=0.0045). As shown in Table 2, higher (P<0.0458) number of blastomeres (47 to 52 cells vs. 62 to 63 cells per blastocyst) was obtained after the addition of aa to P-1 medium supplemented with BSA or hFF than after no addition. A significant (P<0.0008) increase in the number of ICM cells was found after the addition of aa to P-1 medium supplemented with hFF, than after no addition (12 cells vs. 17 cells per blastocyst). However, aa addition to BSA-containing P-1 medium resulted only slight increase in ICM cell number, compared with no addition (14 cells vs. 16 cells per blastocyst; P>0.1). No significant (P=0.1046) increase in the ratio of ICM cell to TE cell (0.32 to 0.36) was obtained after aa addition in both BSA- and hFF-containing media.

Experiment 3: As shown in Table 3, a significant treatment effect was detected in the development of blastocysts to the hatched blastocyst stage (P=0.003). Regardless of the addition of BSA or hFF to P-1 medium, the hatched blastocyst formation was possible only after the addition of aa (11 to 20% vs. 0%, P<0.0492). In the case of the development to the expanded blastocyst stage, no significant (P=0.587) treatment effect was found after the addition of aa to P-1 medium supplemented with BSA or hFF.

DISCUSSION

The promoting effect of aa showing in the results of Experiment 1 was mainly reported by Gardner and his colleagues [8, 9, 15, 23] and the others reported such action of aa in different species of embryos [13, 17, 19, 22, 24]. The detail mechanism of aa during the preimplantation development was reviewed by Van Winkle and Campione [26] and Gardner [11]. It was reported in the bovine [9] that the addition of aa to a chemically defined, protein-free medium stimulates the cytoplasmic maturation of oocytes and that a higher proportion of oocytes cultured in aa-containing medium developed to the pronuclear stage following *in vitro* fertilization. Accordingly, aa is one of essential factors for supporting both *in vitro* growth of oocytes and the preimplantation development of embryos to the blastocyst stage. Based on the previous research outcome, a number of cul-

Table 3. Experimental design and result of Experiment 3: Growth of blastocysts[a] developed from ICR mouse 2-cell embryos cultured in either bovine serum albumin (BSA)- or human follicular fluid (hFF)-containing preimplantation (P)-1 medium supplemented with or without amino acids (aa)

P-1 medium supplemented with	With (+) or without (-) aa	No. of embryos cultured	No. (%)[b] of blastocysts obtained	No. (%)[c] of blastocysts developed to	
				Expanded blastocyst	Hatched blastocyst
BSA	–	38	22 (58)	15 (68)	0 (0)[d]
	+	39	37 (95)	24 (65)	4 (11)[e]
hFF	–	20	11 (55)	6 (55)	0 (0)[d]
	+	23	20 (87)	15 (75)	4 (20)[e]

P value for analyzing the model effect was 0.587 and 0.0003 in expanded blastocyst and hatched blastocyst, respectively.

a) Growth of blastocysts was monitored on 162 hr post-hCG.

b) Percentage of the number of embryos cultured.

c) Percentage of the number of blastocysts cultured.

d,e) Different superscripts within each column are significantly different, $P<0.05$.

ture systems become routinely to use aa for supporting embryo development in various species. For example, improved pregnancy and delivery rates after embryo transfer was obtained from human assisted reproductive technology programs employing aa containing media [4, 10].

The interactions between exogenous aa and other substances present in either BSA and hFF might alter the promoting action of aa on the preimplantation embryo development. It has well been reported that inorganic substrates and proteins, which are present in follicular fluid, affect the action of aa on embryo development [2, 3]. Furthermore, BSA containing numerous unknown factors affects embryo development [5]. In the result of this study (Table 2), aa action on enhancing the proliferation of blastomeres was independent of the presence of BSA or hFF in culture environment, while aa did not promote ICM cell proliferation in BSA-containing medium. These results might reflect the interaction of aa with macromolecules that were added to P-1 medium.

As an energy substrate, pyruvate and lactate were added to P-1 medium instead of glucose. The availability of pyruvate and lactate was already confirmed in the previous studies [3, 11, 13, 24, 25] and it was reported that these substrates act as an energy supplier in various embryo culture media. In the result of this study (Table 1), the promoting effects of aa were not affected by the absence of glucose and by the presence of pyruvate and lactate. So, such results suggest that aa can support preimplantation development of ICR embryos cultured in P-1 medium containing lactate, pyruvate, citrate, taurine and protein macromolecules.

Only limited number of blastocysts developed to the hatched blastocyst stage in the optimized system of this study (Table 3). Furthermore, the ICM cell to TE cell ratio was somewhat lower than that obtained from other studies using different mouse strains [6, 7]. This may be due to the strain specificity or the loss of some ICM cells during the labeling of the blastocyst. However, it can be possible that critical factors responsible for blastocyst hatching are absent. In addition, we employed a continuous culture system for establishing a high embryo per volume ratio, but the accumulation of embryotoxic substances in culture system may be possible. For example, accumulating ammonia of a final product of amino acid metabolism may be responsible for lowing the ratio of ICM cells to trophectodermal cells [6, 12]. An alternative strategy such as the employment of a sequential culture system and the use of culture medium containing the detoxicants for embryotoxin may further promote the growth of blastocysts.

In conclusion, the results of this study demonstrated that aa greatly promoted *in vitro* preimplantation development of ICR mouse 2-cell embryos. Ninety-three to 94% of 2-cell embryos developed to the blastocyst stage after the addition of aa to P-1 medium supplemented with either BSA or hFF. In the results of cytological study, aa mainly exerted on supporting blastomere proliferation during *in vitro* development and it was independent of the presence of hFF and BSA in culture medium.

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