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Amino Acid Concentrations in the Blastocoelic Fluid of *In Vitro*-Produced Bovine Blastocysts

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체외생산된 소 배반포강 내의 아미노산 농도

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요 약

체외생산된 소 배반포 및 탈출 배반포강 내의 유리 아미노산 농도를 측정하였다. 체외배양은 소 혈청알부민이 함유된 합성난관배양액(synthetic oviduct fluid; SOF)에서 실시하였으며 수정 후 180시간의 배반포 및 216시간 후의 탈출 배반포를 실험에 공여하였다. 아미노산 측정은 알부민 대신 polyvinyl alcohol이 함유된 SOF 미소적에 수정란을 분주하고 미세조작기를 이용하여 배반포강 내의 액을 추출하여 20종류의 아미노산을 측정하였다. 탈출 배반포는 isoleucine, leucine 및 methionine 농도가 배반포보다 유의적($p<0.05$)으로 높게 나타났으며, glutamate, aspartate는 두 군간에 차이를 나타내지 않았다. 반면 alanine 및 threamine ($p<0.01$)과 cystine을 제외한 나머지 12종류의 아미노산($p<0.001$)은 배반포가 탈출 배반포에 비해서 유의적으로 높은 측정치를 나타내었다. 비록 glutamine의 경우 배양액 내에 첨가되지 않았으나 두 군, 특히 배반포 내에서 높은 측정치를 보였다. 본 연구결과로 보아 체외생산된 배반포 및 탈출 배반포는 내강에 필수 및 비필수 아미노산을 각기 다른 농도로 함유하고 있는 것으로 사료된다.

(Key words : amino acid, blastocoelic fluid, bovine, blastocyst)

INTRODUCTION

The requirements for energy sources, such as glucose, pyruvate, lactate and amino acids, and for other nutrients, have been investigated in order to clarify their relationships with the viability and developmental capacity of mammalian embryos. By using a chemically defined culture medium, it is possible to evaluate the precise role (s) of different kinds of amino acids in the medium. Amino acids exogenously added to the medium have been shown to affect embryonic development, and their beneficial effects have been found in mouse (Chatot *et al.*, 1989; Gardner and Lane, 1993; Lane and Gardner, 1997; Dumoulin *et al.*, 1992; Dumoulin

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et al., 1997), hamster (Monis and Bavister, 1990; Zhang and Armstrong, 1990; Seshanigri and Bavister, 1991; McKiernan *et al.*, 1995), and cattle(Rosenkrans, and First, 1994; Pinyopummintr and Bavister, 1996; Edwards *et al.*, 1997; Keskintepe *et al.*, 1995) embryos. The uptake and synthesis of amino acids in mouse (Schultz *et al.*, 1981), rabbits (Miller and Schultz, 1987), and cattle (Partridge and Leese, 1996; Lee and Fukui, 1996) have been reported. Amino acids may act as energy substrates, pH regulators, precursors of proteins and nucleic acids, or intracellular osmolytes, and they may improve embryonic development, cell number per embryo, or implantation after transfer (Lane and Gardner, 1997; Van Winkle *et al.*, 1990; Lane and Gardner, 1994). Although it is clear that the uptake of amino acids by embryos varies in different animal species, developmental stages and culture systems, the effects of individual amino acids on the development of embryos are still unknown (McKiernan *et al.*, 1995; Pinyopummintr and Bavister, 1996). Moore and Bondioli (1993) reported that glycine and alanine, either with or without the use of oviductal cells, improved bovine embryonic development. *In vitro*-produced(IVP) bovine embryos were found to deplete aspartate, serine, glutamate, cystine, isoleucine, leucine, arginine and threonine, and to produce alanine (Partridge and Leese, 1996; Lee and Fukui, 1996). The uptake of total amino acids varies in IVP bovine embryos at different stages (Lee and Fukui, 1996).

In mammals, after the compaction of cells to form morulae, the accumulation of the blastocoelic fluid (BF) starts and the blastocoele is formed. Although the biochemistry of the fluid in the blastocoele has been studied in the rabbit (Miller and Schultz, 1987), rat (Brison *et al.*, 1993) and mouse (Brison *et al.*, 1993), there is little information on amino acid contents in the BF of mammalian embryos, especially in IVP bovine blastocysts. Therefore, in the present study, we evaluated the concentrations of 20 amino acids in the BF of bovine blastocysts and hatched blastocysts derived from an IVP system, and further investigated the variation of amino acid concentrations before and after hatching.

MATERIALS AND METHODS

1. *In vitro* maturation (IVM)

Ovaries were obtained from Holstein cows at a local abattoir and were transported to the laboratory in saline(9g/NaCl/l) at 30~35℃ within 2 h. The cumulus-oocyte complexes (COCs) were collected from follicles 2~5 mm in diameter with an 18-gauge needle attached to a 5 ml disposable syringe. Only COCs with an unexpanded cumulus oophorus and evenly granulated cytoplasm were selected and cultured in 4-well plates(Nunclon, Roskide, Denmark) containing 0.5 ml of Tissue Culture Medium (TCM) -199 (Earle' salts with L-glutamine and without sodium bicarbonate; Flow Laboratories Inc., Scotland) supplemented with 10% (v:v) heat-inactivated fetal calf serum (FCS) and 25 mM $NaHCO_3$. The medium was also supplemented with 0.02 AU/ml Antrin (Denka Chemical Co., Ltd.) and 1 μg/ml estradiol-17β, Sigma Chemical Co., U.S.A.). The oocytes were statically cultured for 24 h at 39℃ under a humidified atmosphere of 5% CO_2 in air.

2. *In vitro* fertilization (IVF)

Frozen 0.5-ml straws of semen from three Holstein bulls were thawed at 37℃ and prepared for sperm capacitation. The thawed semen was pooled and layered (0.2 ml) under 1

ml of a modified Tyrode's calcium-free medium (pH 7.4: capacitation medium) in conical tubes (Becton Dickinson Labware, U.S.A.) for a swim-up procedure (Fukui, 1990). The top 0.7 ml of medium was then collected after incubation for 1 h at 39℃. The pooled medium containing spermatozoa was centrifuged (500× g, 10 min), and the pellet was washed twice with the capacitation medium. The final pellet of semen was resuspended in the medium to a concentration of 50×10^6 spermatozoa/ml. An equal volume of the medium containing 200 μg /ml of heparin (Grade 1 from porcine intestinal mucosa: Sigma Chemical, Co, U.S.A.) was added to the semen suspension to yield sperm and heparin concentrations of 25×10^6 cells/ml and 100 μg/ml, respectively. The heparin-treated spermatozoa were incubated for 15 min at 39℃ in 5% CO_2 in air and > 95% humidity.

The IVM oocytes were gently washed 3 times with a modified Tyrode's medium containing 2 mM $CaCl_2$, 2 mM $NaHCO_3$ and 10 mM Hepes (pH 7.4: washing medium). An aliquot (3 μℓ) of the washing medium containing 5 expanding cumulus-enclosed oocytes was placed into a 45 μℓ drop of a modified Tyrode's fertilization medium (pH 7.8) under mineral oil (Sigma Chemical, Co, U.S.A.). Then, 2 μℓ of the heparin-treated semen suspension was added to give a final sperm concentration of 1×10^6 cells/ml. The oocytes and spermatozoa were incubated for 30 h at 39℃ under 5% CO_2 in air.

3. *In vitro* culture (IVC)

A culture medium, synthetic oviduct fluid medium (SOFM; Tervit *et al.*, 1972) containing bovine serum albumin (BSA: 8 mg/ml) was supplemented with 2% (v:v) minimal essential medium (MEM) containing essential amino acids (EAA; 50X, Life Technologies Inc., U.S.A.) and 1% (v:v) MEM containing nonessential amino acids (NEAA; 100X, Life Technologies Inc., U.S.A.). Media were prepared with deionized high quality water (Milli-Q SP. TOC.; Japan Millipore, Japan), sterilized by filtering through a 0.22 μm membrane filter (Gelman Science, U.S.A.), and equilibrated for at least 4 h at 39℃ in 5% CO_2 and 95% air before use.

IVF embryos at the 2- to 4- cell stage were placed in 30 μl drops(5 oocytes or embryos/ drop) in plastic culture dishes (60 × 15 mm; Becton-Dickinson Labware, U.S.A.) under mineral oil and cultured in a humidified atmosphere of 5% CO_2, 7% O_2 and 88% N_2.

4. Aspiration of blastocoelic fluid

IVP blastocysts (180 h of age after insemination) and hatched blastocysts (216 h of age after insemination), derived from the culture in SOFM supplemented with 0.8% (w:v) BSA+ EAA+ NEAA, were washed three times in SOFM (amino acids free) containing 0.1 mg/ml polyvinyl alcohol (PVA, MW 30000~70000; Sigma Chemical, Co, U.S.A.) and transferred to 30 μl drops of SOFM+PVA (15~20 embryos/ drop). A total number of 48 blastocysts and 51 hatched blastocysts with spherical blastocoels were assigned to aspirate. Before aspiration the diameter of the blastocoele of each blastocyst or hatched blastocyst was measured through a scale installed in the eye piece of the microscope. The volume of the BF was then determined by calculating the volume of blastocoele ($V = 3/4\ \pi r^3$, V : volume of blastocoele, r : the average radius of blastocoele). The entire BF was aspirated from blastocysts and hatched blastocysts by micromanipulation through a micro glass pipette (outer diameter : 20~23 μm, inner diameter : 15~20 μm) under a reversed microscope(Nikon, Japan), and the completed disappearance of blastocoels was confirmed after aspiration.

5. Analysis of amino acid concentrations

From each 30 $\mu\ell$ drop of SOFM+PVA containing pooled BF from 15~20 blastocysts or hatched blastocysts, an aliquot of 20 $\mu\ell$ of the medium was taken, diluted 1 : 5 with 0.075 N Li+ buffer (pH 2.97; 7.05 g of trilithium citrate tetrahydrate, 6.5 ml of 60% perchloric acid, 50 $\mu\ell$ of octanoic acid, and 15 ml of methanol contained in 1,000 ml of solution) and frozen at -35℃ until analysis. Free amino acids in the medium were analyzed by a fully automatic amino acid analyzer (JASCO Model 8000 series, Japan; Mikami *et al.*, 1994).

6. Statistical analysis

The concentrations of different amino acids in the BF of IVP blastocysts and hatched blastocysts, and controls (IVC medium only) were compared. Significant differences of amino acid concentrations were analyzed by a MEANS procedure (Student's t-test) in the statistical analysis system (SAS; 1990).

RESULTS

The mean (±s.e.m.) diameters of the blastocoele in both blastocysts and hatched blastocysts were 62±4.5 μm and 116±7.4 μm, respectively. The ranges of the volume of BF in blastocysts (n=20) and hatched blastocysts (n=20) were from 4.1×10^{-5} to 7.2×10^{-5} μl and 38.4×10^{-5} to 53.6×10^{-5} μl, respectively and their mean values with s.e.m. were $5.8\pm0.7\times10^{-5}$ μl and $47.6\pm5.3\times10^{-5}$ μl, respectively.

The concentrations (mM) of amino acids in the BF from blastocysts and hatched blastocysts were compared with those in the IVC medium (Table 1). Alanine, glutamate, glycine, proline, serine and aspartate (NEAA) and methionine (EAA) in the BF from blastocysts, and glutamate and aspartate (NEAA) and isoleucine, leucine and methionine (EAA) in the BF from hatched blastocysts, were found at significantly higher levels ($p<0.05$) than those in the IVC medium. Cystine was not found in the BF from either blastocysts or hatched blastocysts. The concentrations of isoleucine, leucine and methionine were higher ($p<0.05$) in the BF from hatched blastocysts than in the BF from blastocysts, whereas no difference was found in asparagine, aspartate and glutamate concentrations of blastocysts and hatched blastocysts, and threonine, alanine ($p<0.01$) and the remaining 12 amino acids including glutamine were significantly higher in the BF from blastocysts than from hatched blastocysts ($p<0.001$; Table 1). A high concentration of glutamine was found in the BF from both blastocysts and hatched blastocysts, although it was not added to the culture medium.

DISCUSSION

Amino acids are utilized as an energy source and as precursors of synthesis during embryonic development(Van Winkle *et al.*, 1990; Lane and Gardner, 1994; Moore and Bondioli, 1993). When cultured in a medium supplemented with both EAA and NEAA, the development rate, cell number per embryo and pregnancy rate following transfer have been improved in mouse (Mikami *et al.*, 1994), and cattle (Keskintepe *et al.*, 1995; Lee and Fukui, 1996), and the metabolism of amino acids varied in embryos at different stages in many species (Schultz *et al.*, 1981; Bavister and Arlotto, 1990). Moore and Bondioli (1993) reported that NEAA, especially glycine (1.40 mM) and alanine (0.3 mM) were present at high concentrations in the oviductal fluid, and that glycine and alanine added to IVC medium in the absence of oviductal cells improved bovine embryonic development. In the

Table 1. Concentration of amino acids in the blastocoelic fluid of *in vitro*-produced bovine blastocysts and hatched blastocysts

Compound	Concentration(Mean ± SEM : nmol/20μℓ)[a]		
	Medium[b] (mM)	BL[c] (n=48)[e]	HBL[d] (n=51)[e]
Essential amino acids			
1. Arginine	0.6	6.91±0.73^{k}	0±0^{l}
2. Cystine	0.1	0±0	0±0
3. Glutamine	-	7.18±0.77^{k*}	0.46±0.09^{l}
4. Histidine	0.2	2.19±0.23^{k}	0±0^{l}
5. Isoleucine	0.4	3.19±0.34^{g}	12.02±2.41^{h*}
6. Leucine	0.4	7.84±0.84^{g}	39.04±7.83^{h*}
7. Lysine	0.4	2.95±0.32^{k}	0±0^{l}
8. Methionine	0.1	8.96±0.96^{g*}	48.93±9.82^{h*}
9. Phenylalanine	0.2	0.83±0.08^{k}	0±0^{l}
10. Threonine	0.4	3.46±0.37^{i}	0.82±0.16^{j}
11. Typtophan	0.05	-f	-f
12. Tyrosine	0.2	1.75±0.19^{k}	0±0^{l}
13. Valine	0.4	2.43±0.26^{k}	0.44±0.08^{l}
Nonessential amino acids			
14. Alanine	0.1	7.57±0.81^{i*}	2.55±0.51^{j}
15. Asparagine	0.1	0.34±0.03	0±0^{l}
16. Aspartate	0.1	12.10±1.3*	15.25±3.06*
17. Glycine	0.1	7.63±0.08^{k*}	0.23±0.04^{l}
18. Proline	0.1	9.75±1.04^{k*}	0±0^{l}
19. Serine	0.1	3.55±0.37^{k*}	0.27±0.06^{l}
20. Glutamate	0.1	15.29±1.64*	15.64±3.14*

[a] Three replicates.
[b] Concentration of each amino acid in culture medium (SOFM) vs BL and HBL; *p<0.05.
[c] BL; blstocysts, [d]HBL; hatched blastocysts, [e]Number of blastocysts examined, -f: not measured. $^{g-h}$: p<0.05, $^{i-j}$: p<0.01, $^{k-l}$: p<0.001.

rabbit, Miller and Schultz(1987) reported that glycine, alanine, glutamate and serine were present in the oviductal and uterine fluids from day 3 to day 6 after ovulation. Glycine, glutamate and alanine were also found at high concentrations in both the blastocystic cavity and blastocyst cells. Lee and Fukui(1996) reported that EAA combined with NEAA, added to SOFM+PVA, significantly (p<0.01) enhanced the hatching rate of embryos compared to the treatments with EAA or NEAA alone. However, no blastocysts were hatched in the groups without EAA.

In the present study, methionine and glutamine among the EAA, and alanine, aspartate, glycine, proline, serine and glutamate among the NEAA, were significantly higher in the BF of IVP blastocysts than in the IVC medium

(Table 1). It has been shown that NEAA are especially rich in the BF of bovine blastocysts. Lane and Gardner (1997) reported that the NEAA stimulated blastocoelic formation and expansion, and increased the hatching rates of resultant blastocysts. Pinyopummintr and Bavister (1996) compared the development rates of IVP bovine embryos cultured in a chemically-defined protein-free medium (mHECM-3) supplemented with EAA, NEAA, EAA+NEAA, MEM AA, glutamine or 11 AA, and found that higher development rates were obtained when NEAA or glutamine were added. Keskintepe *et al.* (1995) reported that EAA or NEAA alone or together, added to BSA-free SOFM containing citrate, enhanced ($p<0.05$) the developmental capacity of day 7 bovine blastocysts, and NEAA alone showed more ($p<0.05$) benefit to the of embryonic development than other supplementations. The finding in the present study that NEAA and glutamine were high in the BF from both blastocysts and hatched blastocysts, especially in the blastocysts, and indicates that these amino acids may be important biosynthetic substrates and energy sources for embryonic development in bovine IVP blastocysts. Gardner and Lane (1993) suggested that NEAA with or without glutamine increased cell numbers but that EAA without glutamine resulted in a lower cell number. Lee and Fukui (1996) found that glutamine was produced by IVP bovine blastocysts. As glutamine was not added into the culture medium, the present result confirms that bovine blastocysts produce glutamine. Taken together, these data suggest that glutamine may play an important role in the development and hatching of bovine embryos. Arginine, histidine, lysine, phenylalanine, tyrosine, asparagine and proline were not found in the BF of hatched blastocysts, and they were also present only at low concentrations in the BF of blastocysts. It appears that those amino acids were utilized and/or depleted by the embryos during hatching.

As shown in Table 1, 6 EAA (isoleucine, leucine, methionine, glutamine, threonine and valine) and 5 NEAA (alanine, aspartic, glutamic, glycine and serine) were found in the BF of hatched blastocysts, and the concentrations of isoleucine, leucine and methionine (EAA) were higher ($p<0.05$) in the BF of hatched blastocysts than in the BF of blastocysts. As companed with the amino acid concentrations in the IVC medium, glutamate and aspartate (NEAA) and isoleucine, leucine and methionine (EAA) were found at significantly higher levels in the BF from hatched blastocysts. These findings may indicate that glutamate, aspartate, isoleucine, leucine, and methionine could be related to the hatching process of bovine IVP blastocysts. Lane and Gardner (1997, 1994) reported that EAA stimulated the development of inner cell mass (ICM) cells of cultured mouse precompaction embryos and that EAA increased fetal development. Although it is believed that both EAA and NEAA are necessary for the hatching of blastocysts, some amino acids may have specific inhibitory or stimulatory functions. Bavister and Arlotto (1990) reported the effects of single amino acids in hamster embryos and suggested that several amino acids (glycine, cystine and lysine) are stimulatory, while others (proline, serine, threonine, histidine, alanine, leucine, aspartate and methionine) are neutral, or even inhibitory (phenylalanine, valine, isoleucine, tyrosine, tryptophan and arginine). Mckiernan *et al.* (1995) also found that glutamine, aspartate, serine, histidine, glycine, taurine, and proline were stimulatory and cysteine, leucine, tyrosine, valine, methionine, phenylalanine, isoleucine, arginine, and tryptophan were inhibitory. We,

therefore, consider that the 11 amino acids presenting in the BF of hatched blastocyst may play a role as stimulators for the hatching of bovine IVP embryos.

Partridge and Leese (1996) compared amino acids taken up by *in vivo*- and *in vitro* derived bovine embryos, and reported that aspartate, glutamate and threonine were significantly depleted, while alanine was produced at a significant rate. Lee and Fukui (1996) reported that aspartate, serine, glutamate, cystine, isoleucine, leucine and arginine were depleted and alanine was produced at significant rates by day 7 bovine blastocysts. In the present study, all amino acids except cystine were shown to exist in the BF of IVP bovine blastocysts. Brison *et al.* (1993) reported that the concentrations of glucose, L-lactate and pyruvate in the BF of mice and rats were 2.30 and 2.75 mM, 14.6 and 19.6 mM, and 0.13 and 0.50 mM, respectively. Dizio and Tasca (1977) and Rieger *et al.* (1992) also reported that the bovine blastocoelic expansion was accompanied by significant increases in the metabolism of glucose and glutamine, presumably reflecting the increased energy demands of the Na+/K+ ATPase which are probably necessary for formation and maintenance of the blastocoele.

In conclusion, the present results indicate that IVP bovine BF contains several EAA and NEAA, and there is significant differences in the amino acid concentrations in the BF between blastocysts and hatched blastocysts produced by the *in vitro* system.

SUMMARY

Concentrations of free amino acids in the BF of IVP bovine BL and HBL were examined in this study. The embryos derived from IVF oocytes were cultured in a SOFM containing BSA, EAA and NEAA. BF was aspirated from BL (180 h of age after insemination) and HBL (216 h of age after insemination), and introduced into drops of SOFM (30 μl/drop) containing PVA through micromanipulation. The medium containing BF was then subjected to measurement of 20 amino acids by an automatic amino acid analyzer. The concentrations of isoleucine, leucine and methionine were higher ($p<0.05$) in the BF from HBL than from BL, and no difference was found in aspartate or glutamate concentrations between BL and HBL, while threonine, alanine ($p<0.01$) and the rest of the amino acids ($p<0.001$) were significantly higher in the BF from HBL than from BL. Cystine was not found in either BL or HBL. A high concentration of glutamine was found in the BF from both BL and HBL, although it was not added to the culture medium. These results indicate that bovine BF contains several EAA (methionine in BL and isoleucine, leucine and methionine in HBL) and NEAA (alanine, glutamate, glycine, proline, serine and aspartate in BL, and glutamate and aspartate in HBL), and there is significant differences in the amino acid concentration in the BF between BL and HBL derived by IVP.

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