

Effects of Amino Acids on the In Vitro Development of Mongolian Gerbil (*Meriones unguiculatus*) Embryos

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Abstract: Mongolian gerbil 1-cell embryos can develop into blastocysts in vitro in a co-culture with oviductal cells in mTCM199 medium. When 1-cell embryos are cultured in a chemically defined medium, they do not develop into blastocysts. Thus, the objective of this study was to examine the effects of amino acids on the in vitro development of Mongolian gerbil embryos. Amino acid depletion/accumulation by the Mongolian gerbil preimplantation embryo was analyzed with an amino acid analyzer. The amino acids of threonine, valine, isoleucine, leucine, lysine, serine, glutamine, glycine, and alanine were depleted in the medium at all the developmental stages. Two-cell stage embryos were cultured in mM16 medium supplemented with each amino acid. We found that 37.1% and 11.4% of 2-cell embryos reached the morula and blastocyst stages, respectively, after supplementation of the medium with valine. Three amino acids (threonine, aspartic acid, and glutamine) promoted development to the 4-cell stage ($P < 0.05$). Although the combination of valine and threonine, aspartic acid, or glutamine significantly increased development to the 4-cell and 8-cell stages, no improvement of the blastocyst rates was observed. This study has demonstrated that amino acids can support preimplantation gerbil embryo development in vitro.

Key words: Amino acid, Culture, Embryo, Mongolian gerbil, Valine

Introduction

Unlike Mongolian gerbil (*Meriones unguiculatus*) 8-cell stage embryos, 1-cell stage embryos cannot

develop to the blastocyst stage in chemically defined media [1]. We reported that Mongolian gerbil 1-cell stage embryos can develop to blastocysts in co-culture with oviductal cells in TCM199 medium supplemented with pyruvate, lactate, and fetal calf serum [2]. The improvement of culture media by adding biochemical substrates to support the development on Mongolian gerbil embryos to the blastocyst stage greatly increases the potential of this experimental animal in developmental biology and biomedical research.

Amino acids play a multitude of roles during early embryonic development. In addition to protein biosynthesis, they also stimulate activation of the embryonic genome and contribute to energy production, osmoregulation, pH control, cell homeostasis, and signal transduction cascades [3]. Even a brief (5 min) exposure of fertilized mouse embryos to a medium lacking amino acids is detrimental to blastocyst development and the total cell number of the blastocysts [4]. Several studies have demonstrated that the addition of pooled amino acids facilitates the development of bovine [5–7], murine [8–9], ovine [10], rat [11–12], rabbit [13], human [14–15], hamster [16–17], and porcine [18–19] embryos produced by *in vivo* or *in vitro* fertilization.

Partridge and Leese [20] showed that amino acid depletion/accumulation by embryos in culture media varies between species. A notable feature of bovine embryo amino acid metabolism is the accumulation of alanine in increasing amounts throughout the development process. As a first approach to understanding the mechanisms by which mixtures of amino acids promote the preimplantation development of Mongolian gerbil embryos, we examined the depletion/accumulation of amino acids in the culture medium containing Mongolian gerbil embryos co-cultured with oviductal cells. It should be emphasized

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that this approach was not intended to constitute a strict kinetic analysis of amino acid uptake; rather, it was intended to determine the way in which embryos modify the composition of mixtures of amino acids present at physiological concentrations. The effects of amino acids on embryonic development have been investigated in some species, but they vary from species to species and there is no information available for Mongolian gerbils. Thus, the objectives of the present study were i) analysis of amino acid depletion/accumulation in the medium by Mongolian gerbil embryos at different developmental stages in co-culture with oviductal cells, and ii) determination of the effects of amino acid supplementation on the *in vitro* preimplantation development of Mongolian gerbil embryos.

Materials and Methods

Animals

Ten- to fourteen-week-old adult virgin female Mongolian gerbils were used at random stages of their estrous cycles. These animals were maintained and experimented on in accordance with the Guidelines for Regulation of Animal Experimentation, Faculty of Agriculture, Shinshu University. They were maintained under controlled light conditions (12 h light, 12 h darkness) and allowed free access to a pellet diet and tap water. Mongolian gerbils were intraperitoneally injected with 20 IU of pregnant mare serum gonadotropin (Peamex, Sankyo Ltd., Japan) 54 h before an intraperitoneal injection of 20 IU human chorionic gonadotropin (hCG; Puberogen, Sankyo Ltd, Japan). After the hCG injection, the animals were mated overnight with fertile males of the same colony.

Experiment 1: Analysis of changes in amino acid concentrations in culture medium containing Mongolian gerbil embryos at different developmental stages co-cultured with oviductal cells

A co-culture system with oviductal cells in a culture medium was employed for this study, because Mongolian gerbil early stage embryos cannot develop to the blastocyst stage *in vitro* in a chemically defined medium. Tissue Culture Medium 199 (Medium 199; Sigma, St. Louis, MO, USA) with 10 % fetal calf serum (FCS; Gibco-BRL, Grand Island, NY, USA), 0.5 mM sodium pyruvate, and 20.4 mM sodium lactate was used as the culture medium (mTCM199). Embryos at 8-cell, morula, and blastocyst stages were recovered by flushing the oviducts with mTCM199 culture medium 73,

113, and 133 h, respectively, after the hCG injection. Oviductal cells were collected by lightly scraping the luminal surface with a scalpel 73, 113, and 133 h after the hCG injection and were then suspended in mTCM199 in a 4-well Multi-dish (Nunc, Roskilde, Denmark). The embryos were co-cultured for 12 h with oviductal cells in a 4-well Multi-dish with 1 ml of mTCM199 under a humidified atmosphere of 5 % CO₂ in air at 37°C. The control medium was a medium containing only oviductal cells collected 73, 113, and 133 h after the hCG injection, and cultured for 12 h under a humidified atmosphere of 5 % CO₂ in air at 37°C.

Amino acid analysis

The culture medium was collected after 12 h of culture, its protein content was removed, and it was diluted 1:1 with 5 % 5-sulfosalicylic acid dihydrate (Wako, Osaka, Japan) solution. Amino acids were analyzed with a L-8800 high-speed amino acid analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). Values were obtained for each amino acid by subtracting the value for the amino acid in co-culture medium containing the embryo and the oviductal cells from that for the amino acid in the medium containing only oviductal cells.

Experiment 2: Effects of amino acids on in vitro development of Mongolian gerbil embryos

Modified medium 16 was used in this experiment. The composition of Medium 16 is 99 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂, 1.19 mM MgSO₄·7H₂O, 25 mM NaHCO₃, 0.33 mM Na-pyruvate, 23.3 mM Na-lactate, 2 mM glucose, 4 mg/ml bovine serum albumin-V; (Sigma, St. Louis, MO, USA), 100 U/ml penicillin, 50 µg/ml streptomycin, and 100 µM ethylenediaminetetraacetic acid. It was modified adding 2 mM glucose, removing KH₂PO₄, and adjusting the osmolarity to 290 mOsmol by altering the amount of NaCl. Osmolarity was determined by freezing point depression with an Advanced micro-osmometer (Advanced Instruments Inc., Norwood, MA, USA). Modified M16 (mM16) was supplemented with a single amino acid at a concentration of 0.2 mM, as described previously [12]. Two-cell stage embryos were collected by flushing the oviducts with mM16 43 h after the hCG injection. Droplets of the culture medium were covered with mineral oil in a polystyrene culture dish (35 mm × 10 mm) and kept in a CO₂ incubator (humidified air with 5 % CO₂, 37°C) for 3–5 h before use. Embryos were cultured in CO₂ incubator (humidified air with 5 % CO₂, 37°C).

Amino acid abbreviations

His, histidine; Thr, threonine; Met, methionine; Val, valine; Trp, tryptophan; Phe, phenylalanine; Ile, isoleucine; Leu, leucine; Lys, lysine; Asp, aspartic acid; Asn, asparagine; Glu, glutamic acid; Ser, serine; Gln, glutamine; Gly, glycine; Arg, arginine; Ala, alanine; Tyr, tyrosine; Pro, proline; Cys, cysteine.

Cell count

To count the cell number of the blastocysts developed in mM16 supplemented with valine and those generated *in vivo*, the embryos were selected and incubated in 300 μ l of PBS with 1 % Triton X and 100 μ g/ml propidium iodide (Sigma, St. Louis, MO, USA) for up to 30 seconds. The blastocysts were immediately transferred into 300 μ l of fixative containing anhydrous ethanol with 25 μ g/ml Hoechst 33342 (Sigma, St. Louis, MO, USA) and stored at 4°C overnight. The stained embryos were immersed in glycerol and mounted on a slide. Then, the embryos were observed by fluorescence microscopy to count the cell numbers of ICM (blue) and trophoblast (red). The experiment was repeated 5 times.

Experiment 3: Effects of combinations of amino acids on *in vitro* development of Mongolian gerbil 2-cell stage embryos

Modified M16 containing bovine serum albumin-V was supplemented with 0.2 mM valine and 0.2 mM aspartic acid, 0.2 mM valine and 0.2 mM threonine, or 0.2 mM valine and 0.2 mM glutamine. The methods used are described in detail elsewhere (Exp. 2).

Statistical analysis

The Number Cruncher Statistical System (NCSS; NCSS Statistical Software, Kaysville, UT, USA) Version 5.01 computer software package was used for all statistical analyses. Embryo developmental rates were subjected to arc-sine transformation before statistical analysis. Amino acid depletion and accumulation were expressed as pmol per embryo per hour. All data are presented as mean \pm S.E.M. Differences between groups were tested by Student's t-test or, in the case of those characterized by non-parametric distributions upon transformation (as assessed by Anderson-Darling tests), by the Mann-Whitney U test. Differences were considered to be statistically significant at $P < 0.05$.

Results

Experiment 1: Analysis of changes in amino acid concentrations in culture medium containing Mongolian gerbil embryos at different developmental stages co-cultured with oviductal cells

The amino acids, depletion/accumulation in the culture medium by Mongolian gerbil oviductal cells at three different stages of embryonic development is shown in Fig. 1. Among the amino acids, histidine, threonine, isoleucine, leucine, lysine, aspartic acid, glutamic acid, glutamine, glycine, arginine, tyrosine, and proline were depleted at all three stages. Alanine and cysteine accumulated at all three stages. Only in the cases of phenylalanine and glutamine were there significant differences among the three stages. No marked changes (depletion or accumulation) were observed for methionine, valine, tryptophan, and serine.

The amino acid depletion/accumulation in the culture medium by Mongolian gerbil embryos during preimplantation development is shown in Fig. 2. Among the amino acids, threonine, valine, isoleucine, leucine, lysine, serine, glutamine, glycine, alanine, and proline were significantly depleted in the medium at the 8-cell, morula, and blastocyst stages. A difference in concentration among the developmental stages was observed for aspartic acid: the concentration increased at the 8-cell and morula stages and decreased at the blastocyst stage ($P < 0.05$). The accumulation of glutamic acid was observed in the medium only at the morula stage ($P < 0.05$). No marked change (depletion or accumulation) was observed for histidine, methionine, tryptophan, arginine, tyrosine, or cysteine.

Experiment 2: Effects of amino acids on *in vitro* development of Mongolian gerbil 2-cell stage embryos

The effects of single amino acids on the development of 2-cell stage embryos are shown in Table 1. In the presence of valine, 37.1 and 11.4 % of the embryos developed to the morula and blastocyst stages, respectively. Development to the 8-cell stage was observed after supplementation with threonine, tryptophan, methionine, valine, asparagine, aspartic acid, glutamic acid, glutamine, serine, glycine, and alanine, but these rates of development were significantly lower than that of the control (without amino acids). Three amino acids (leucine, lysine, and phenylalanine) enhanced only development to the 4-cell stage. Four others (isoleucine, histidine, arginine, and proline) showed no enhancement of development. Three amino acids (threonine, aspartic acid, and

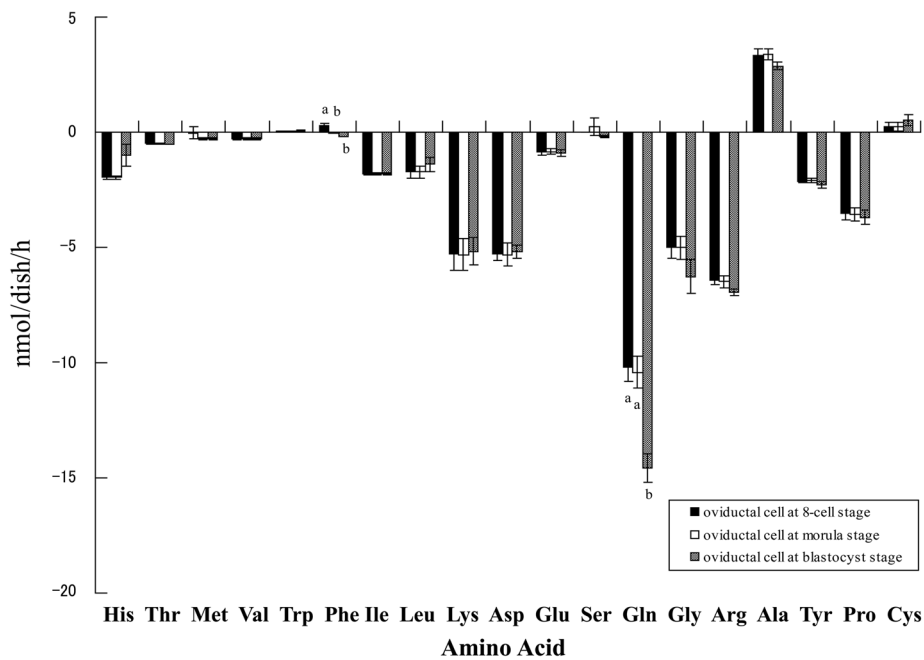


Fig. 1. Amino acid depletion/accumulation by Mongolian gerbil oviductal cells. Values are expressed as mean \pm SEM. Different superscripts for the same amino acid denote significant differences ($P < 0.05$).

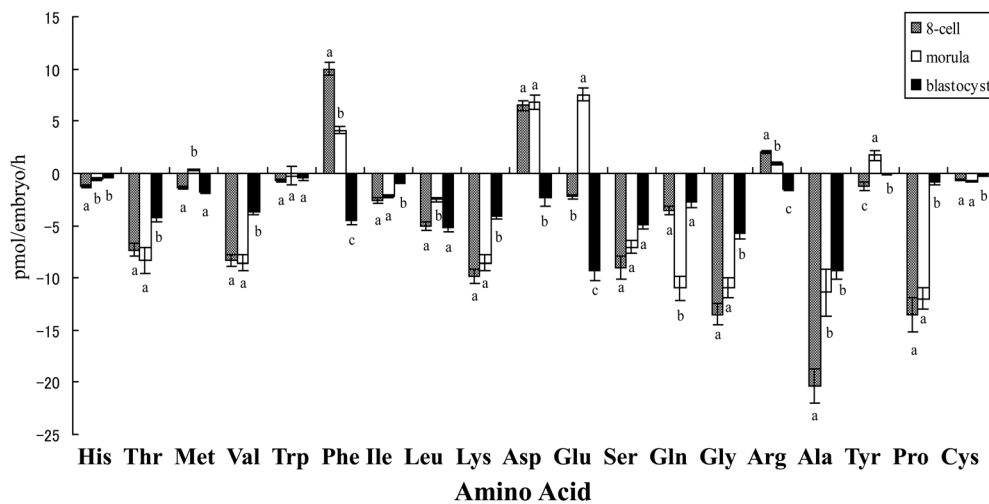


Fig. 2. Amino acid depletion/accumulation by Mongolian gerbil preimplantation embryos. Values are expressed as mean \pm SEM. Twenty embryos were cultured per sample and the experiment was repeated 8 times. Different superscripts for the same amino acid denote significant differences ($P < 0.05$).

glutamine) showed higher developmental rates to the 4-cell stage than that of the control.

Cell count

A blastocyst stained with Hoechst 33342 and PI is

shown in and Fig. 3. Compared to development *in vivo*, supplementation of valine to the culture medium tended to increase the cell numbers (mean \pm SEM) of total (50.0 ± 1.6 , vs 54.5 ± 1.3), ICM (13.7 ± 1.0 , vs 17.0 ± 1.1), and TE (36.5 ± 1.3 , vs 37.5 ± 1.2) (Fig. 3).

Table 1. Effects of single amino acid supplementation on development of Mongolian gerbil embryos

Amino acid	No. (%) of 2-cell stage embryos developing to				
	N	4-cell	8-cell	Morula	Blastocyst
–	50	44 (88.0) ^a	37 (74.0) ^a	0	0
Threonine	46	44 (95.7) ^b	20 (43.5) ^c	0	0
Tryptophan	56	36 (64.3) ^d	24 (42.9) ^c	0	0
Isoleucine	50	0	0	0	0
Leucine	48	4 (8.3) ^h	0	0	0
Lysine	60	6 (10.0) ^h	0	0	0
Phenylalanine	62	20 (32.3) ^g	0	0	0
Histidine	60	0	0	0	0
Methionine	52	28 (53.8) ^e	20 (38.5) ^d	0	0
Valine	70	55 (78.5) ^c	40 (57.1) ^b	26 (37.1)	8 (11.4)
Asparagine	42	23 (54.8) ^e	15 (35.7) ^d	0	0
Aspartic acid	60	58 (96.7) ^b	28 (46.7) ^c	0	0
Glutamic acid	56	49 (87.5) ^a	10 (17.9) ^f	0	0
Glutamine	55	52 (94.5) ^b	49 (89.1) ^b	0	0
Serine	52	34 (65.4) ^d	10 (19.2) ^f	0	0
Glycine	49	37 (75.5) ^c	14 (28.6) ^e	0	0
Arginine	54	0	0	0	0
Alanine	54	24 (44.4) ^f	9 (16.7) ^f	0	0
Tyrosine	44	4 (9.1) ^h	0	0	0
Proline	56	0	0	0	0
Cysteine	42	10 (23.8) ^g	0	0	0

a–h: Different superscripts within the same column denote significant differences ($P < 0.05$). Experiment was repeated 4 times.

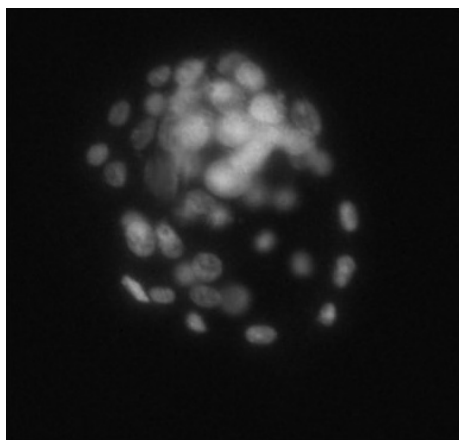


Fig. 3. A blastocyst developed in culture medium supplemented with valine stained with Hoechst 33342 and PI to determine its cell number.

Experiment 3: Effects of combinations of valine and each of the other amino acids on *in vitro* development of Mongolian gerbil 2-cell stage embryos

The effects of combinations of valine and each of the other amino acids on the development of 2-cell stage

embryos are shown in Table 2. All the combinations of valine significantly promoted development to the 4-cell and 8-cell stages, and development to the blastocyst stage was achieved by supplementing valine with asparagine, or glutamine.

Discussion

It is the main finding of this study that the development of Mongolian gerbil embryos in culture can be regulated by supplementation of the culture medium with specific amino acids. Amino acids at very high concentration levels have been detected in mammalian oviductal fluid [21], and the concentrations of these amino acids are far in excess of the metabolic requirements of mammalian preimplantation embryos; therefore, they must have other roles in embryonic development. Our finding is in agreement with the result of Bavister and Arlotto [22] and Gardner and Lane [23], who found that specific amino acids either enhance or suppress the early embryonic development *in vitro* in the hamster and mouse, respectively.

Our result indicates that amino acid concentrations in the culture medium change markedly, and that the degree of change varies throughout the developmental

Table 2. Effects of combinations of valine and each of the other amino acids on embryo development

Combinations of valine and	No. (%) of 2-cell stage embryos developing to				
	n	4-cell	8-cell	Morula	Blastocyst
Aspartic acid	64	62 (96.9) ^a	56 (87.5) ^a	53 (82.8) ^b	4 (6.3) ^a
Glutamine	60	57 (95.0) ^a	56 (93.3) ^a	14 (23.3) ^c	4 (6.7) ^a
Threonine	63	59 (93.7) ^a	49 (77.7) ^b	28 (44.4) ^a	0
None ¹⁾	70	55 (78.5)	40 (57.1)	26 (37.1)	8 (11.4)

¹⁾The same values cited for valine in Table 1. a–c: Different superscripts within the same column denote significant differences ($P < 0.05$). Experiment was repeated 5 times.

stage. Protein synthesis patterns could underlie the developmental changes in the net rates of amino acid depletion/accumulation. In the present study, one potential predictor of blastocyst formation was the pattern of amino acid accumulation/depletion. The results show that a higher amount of amino acid depletion occurred at the morula stage. There was a marked variation in the concentration of all amino acids at the different developmental stages. The results of Houghton *et al.* [24] and Brison *et al.* [25] indicate that the patterns of depletion/accumulation of individual amino acids by preimplantation embryos vary at different rates. This suggests that amino acid requirements change during embryonic development. In addition, amino acids exhibited significantly different patterns of amino acid depletion/accumulation between arresting and developing embryos suggesting that amino acids play different roles at different stages of embryonic development. We observed that threonine, valine, lysine, serine, glutamine, glycine, alanine, and proline were markedly depleted in the medium, while very little depletion was observed for histidine and tryptophan. Paradoxically, Spindle and Pedersen [26] found that the omission of histidine, methionine, threonine, tryptophan, or valine from the medium significantly inhibited the development of embryos. Subsequently, Booth *et al.* [27] observed depletion of leucine and threonine in a culture medium designed for porcine embryos. In our study phenylalanine accumulated in the medium. Its accumulation must be studied further to obtain better understanding of this phenomenon.

In our study glycine, alanine, and proline were markedly depleted in the culture medium with embryos at the 8-cell to blastocyst stages. Accumulation of aspartic acid and arginine occurred at the 8-cell and morula stages, while depletion occurred at the blastocyst stage. These findings are in agreement with

the result that glutamine was depleted from the culture medium at various developmental stages of porcine embryos [27]. With regard to amino acid depletion, our most interesting findings was the marked depletion of alanine at all observed stages of development. This finding is in agreement with the result that alanine depletion in bovine embryos was observed at all the developmental stages [20]. Van Winkle and Dickinson [28] hypothesized that alanine may enable embryos to sequester waste nitrogen found in murine blastocysts, resulting in metabolic perturbations and changes in gene expression. Aspartic acid, glutamic acid, threonine, and leucine were depleted significantly by *in vitro* derived bovine blastocysts [20].

Our study has revealed that amino acids have different stage-specific roles in the development of Mongolian gerbil early stage embryos. Amino acids act as novel signaling elements in insulin-sensitive tissues [29–33]. Because early embryos contain insulin-sensitive cells [34], they probably also respond to amino acid signaling molecules. Many inhibitory effects of amino acids have been attributed to the ammonia that is produced when amino acids are metabolized by embryos or spontaneously break down in culture at specific temperatures [23, 35–37]. The results of our present study show that among the amino acids, threonine, glutamine and valine significantly enhanced the development of Mongolian gerbil 2-cell embryos beyond the 4-cell stage. Our most remarkable finding is that valine is the only amino acid that enhanced the development up to the morula and blastocyst stages of Mongolian gerbil 2-cell stage embryos. Although valine enhanced development to the morula and blastocyst stages, it was not so effective for development to the 8-cell stage. Note that valine was shown to be necessary for the net protein accumulation that occurs in mouse blastocysts *in vivo* during the 10 h period preceding implantation [38]. In the present study, a large amount

of valine was depleted from the culture medium during the morula and blastocyst stages. Isoleucine, methionine, and valine are branched-chain glucogenic amino acids whose carbon skeletons are degraded to succinyl-CoA, fumarate, or oxaloacetate, all of which are ultimately capable of producing energy by entering the TCA cycle [39]. Thus, it is possible that valine promotes blastocyst development by generating energy in the Mongolian gerbil.

Eagle's non-essential amino acid mixture (alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, and serine) increases the rate and frequency at which blastocysts are formed from the 1-cell stage in the mouse [23, 40]. Moreover, the implantation rate of such mouse blastocysts upon transfer to surrogate mothers is greater when Eagle's non-essential amino acids are present in the medium during preimplantation development *in vitro* [39, 40]. Even brief exposure of approximately 5 min to a medium not containing Eagle's non-essential amino acids reduces significantly the proportion of mouse zygotes developing into morulae and blastocysts *in vitro* and the total number of cells in those blastocysts [8]. Specifically, it was established that Eagle's non-essential amino acids increase the cleavage rate of the first three cell divisions and enhance blastocoel development and hatching in mouse embryos [23, 41, 42]. In contrast, Eagle's essential amino acids (arginine, cysteine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine) do not increase the rate of cleavage until after the 8-cell stage when development of the inner cell mass takes place in the mouse [42]. Our observations in this study indicate that aspartic acid, glutamine, and threonine efficiently facilitated the development of 2-cell stage embryos to the 4-cell stage.

The final part of this study was concerned with how combinations of amino acids affect the development of Mongolian gerbil 2-cell stage embryos. Our findings suggest that the combination of valine with aspartic acid or glutamine supported the embryonic development up to the blastocyst stage, i.e. aspartic acid and glutamine can induce development to the blastocyst only in combination with valine. Although the maximum rates of development to the 8-cell and morula stages were observed in the medium with valine and glutamine, and valine and aspartic acid, respectively, the rate of blastocyst development was almost identical for these two combinations.

In conclusion, this study demonstrated that amino acids promote preimplantation Mongolian gerbil embryo

development *in vitro*. It is probable that each amino acid affects the early embryonic development, e.g., by affecting osmolarity, by regulating pH, or by acting as energy substrates. The roles of medium components and their interactions with amino acids warrant further investigation to obtain a more complete understanding of embryonic development and its control *in vitro*.

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