ANIMAL EXPERIMENTATION

Effect of Essential Amino Acids on Mouse Embryo Viability and Ammonium Production

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Purpose: To examine the effect of essential amino acids concentrations on mouse embryo development.

Methods: Mouse embryos were cultured in medium with different concentrations of essential amino acids and development to the blastocyst stage and viability assessed. Ammonium production resulting from medium breakdown and amino acid metabolism by embryos were also assessed.

Results: Reducing the essential amino acid concentration significantly increased blastocyst development and cell numbers. Lowering the essential amino acid concentration decreased ammonium production in the medium.

Conclusions: Culture media for the development of preimplantation embryos should have a reduced essential amino acid concentration to facilitate embryo development.

KEY WORDS: amino acids; culture; development; mouse.

INTRODUCTION

Addition of amino acids to the culture medium for mammalian embryos has been shown to stimulate development (1–9). However, there appears to be differential regulation of embryo development by amino acids. The amino acids classified as nonessential for somatic cells by Eagle (10) have been shown to stimulate development of the cleavage stage embryo as well as stimulating blastocoel formation in the postcom-

paction stage embryo (1,7,11). Interestingly, those amino acids classified as nonessential are the amino acids that are present at relatively high concentrations in the female reproductive tract (12). In contrast, the amino acids classified by Eagle (10) as essential for somatic cells in culture are present in the reproductive tract at low concentrations (12). Addition of these essential amino acids to the culture medium inhibits development of cleavage stage embryos in vitro (1). However, essential amino acids stimulate the development of the inner cell mass (ICM) of cultured blastocysts and therefore enhance fetal development after transfer (7,13). Importantly, the concentrations of essential amino acids as described by Eagle (10) are significantly higher than the embryo would be exposed to in vivo. It has recently been demonstrated that reducing the concentration of essential amino acids for development of the cow embryo in vitro increased blastocyst development (14). However, the viability of these embryos was not assessed.

When culturing embryos in media supplemented with amino acids it is necessary to replace the culture medium every 48 h due to the production of embryo-toxic ammonium from both the spontaneous breakdown of amino acids and from amino acid metabolism (1). When amino acids are present in the medium at the concentrations described by Eagle (10) the rate of ammonium production was such that an inhibitory concentration was reached in less than 48 h of culture (1). Therefore, reducing the essential amino acids concentration should result in a reduction of ammonium generation in the culture medium and further enhance embryo development.

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Table I. Concentrations of Essential Amino Acids in the Culture Media

Amino acid	Eagle's medium (mM)	1/2 Eagle's medium (mM)	1/4 Eagle's medium (mM)	1/8 Eagle's medium (mM)
Arginine	0.6	0.3	0.15	0.075
Cystine	0.1	0.05	0.025	0.0125
Histidine	0.2	0.1	0.05	0.025
Isoleucine	0.4	0.2	0.1	0.05
Leucine	0.4	0.2	0.1	0.05
Lysine	0.4	0.2	0.1	0.05
Methionine	0.1	0.05	0.025	0.0125
Phenylalanine	0.2	0.1	0.05	0.025
Threonine	0.4	0.2	0.1	0.05
Tryptophan	0.5	0.25	0.125	0.0625
Tyrosine	0.2	0.1	0.05	0.025
Valine	0.4	0.2	0.1	0.05

Note. In all media nonessential amino acids were present at 0.1 mM and alanylglutamine at 1.0 mM.

The aim of this study was therefore to examine the effect of essential amino acid concentration in embryo culture medium and to assess development at the blastocyst stage by determining ICM formation and fetal development after transfer. Additionally, the ammonium generation in the presence of different concentrations of essential amino acids was quantitated.

MATERIALS AND METHODS

Media

The medium for embryo collection and manipulation was a HEPES buffered modification of medium G1.2 (H-G1.2), modified by omitting EDTA and replacing 20 mM NaHCO₃ with 20 mM HEPES and adjusting to pH 7.35. Media for embryo culture were G1.2 and G2.2 supplemented with 5 mg/ml human serum albumin as the sole protein source (13,15–17). All media were prepared weekly from stock solutions and stored at 4°C. All salts were analar grade and purchased from BDH (Dorset, UK). Pyruvate, lactate, taurine, alanylglutamine and HEPES were purchased from Sigma Chemical Co. (St. Louis, Mo). Amino acid and vitamin solutions were obtained from ICN (Aurora, OH). EDTA and HSA were obtained from Vitrolife AB (Gothenburg, Sweden).

Embryo Collection

Embryos were collected from 4-week-old CF1 female mice. Multiple ovulations were induced by an i.p. injection of 5 iu Pregnant Mare's Serum Gonadotropin (Sigma) followed later by 5 iu of human Chorionic Gonadotrophin (hCG; Sigma). After

the second injection females were placed with males of the same strain to obtain pure CF1 embryos. Zygotes were collected at 21 h post-hCG in H-G1.2 and denuded by incubation with hyaluronidase (0.5 mg/ml, Sigma., bovine testes, type IV) for less than 1 min. Embryos were washed twice in H-G1.2 and once in medium G1.2 before placement in culture.

Embryo Culture

Embryos were cultured in 20 µl drops of medium under mineral oil (Sigma). All embryos were cultured in medium G1.2 at 37°C in 6% CO₂:5% O₂: 89% N₂ for 48 h to around the 8-cell stage. After 48 h of culture, embryos were washed well in medium G2.2 and then cultured in G2.2 with the appropriate concentrations of Eagle's essential amino acids (Table I) for a further 48 h to the blastocyst stage.

Allocation of Cells to the Inner Cell Mass and Trophectoderm

Allocation of cells in blastocysts to the inner cell mass and trophectoderm was assessed using a modification of the technique reported by Hardy *et al.* (18), as described by Gardner *et al.* (19).

Embryo Transfer

Blastocysts were transferred to Day 4 pseudopregnant F1 female mice (–1 day asynchronous). Embryos from each treatment were randomly allocated to each uterine horn using random numbers. Five blastocysts were transferred to each uterine horn. On Day 15 of pregnancy, females were sacrificed and implantation

sites and numbers of fetuses determined. Weights of resultant fetuses were also assessed.

Ammonium Assay

Ammonium levels in the medium and ammonium production by embryos were assessed using an ultramicrofluorometric technique (1).

The reaction reagents had the following composition: 0.24 mM NADH, 0.75 mM NaHCO₃, 0.63 mM ADP, 14.15 mM α -ketoglutarate, 3 U glutamate dehydrogenase/ml (EC 1.4.1.3) in 157 mM triethanolamine buffer, pH 8.0. A calibration curve of ammonium chloride concentrations between 0 and 0.5 mM was run with each series of assays.

For determination of ammonium concentration in the culture medium, a 40 nl sample of medium was removed every 24 h. For analysis of ammonium production, individual blastocysts were placed in 20 nl of medium G2.2 with the appropriate essential amino acid concentrations for 4 h. End point analysis of the ammonium production was measured by determining the increase in ammonium concentration compared with control medium incubated without embryos.

Statistical Analysis

Differences in embryo development in culture and embryo viability were assessed using linear-logistic regression where the error distribution was assumed to be binomial. The null hypothesis of no treatment effect against a treatment effect was tested using the log-likelihood ratio statistic. The day of experiment was fitted as a factor. For embryo viability studies, the recipient female was also fitted as a factor. A value

of $P < .05$ was considered to be significantly different. Cell numbers and ammonium production were assessed using ANOVA followed by Bonferroni procedure for multiple comparisons.

RESULTS

Effect of Essential Amino Acid Levels on Embryo Development in Culture

Zygotes were cultured for 48 h to the 8-cell stage in medium G1.2 and then transferred to one of 5 treatments; G2.2 (contains essential amino acids at the concentrations described by Eagle (10)), with nonessential amino acids and alanylglutamine or G2.2 where the essential amino acids were reduced to either $\frac{1}{2}$, $\frac{1}{4}$, or $\frac{1}{8}$ of the original concentration (Table I) or to medium G2.2 without essential amino acids.

Addition of essential amino acids to the medium at half the concentration did not affect development to the blastocyst stage (Table II), although the percentage of blastocysts that were hatching was increased (Table II). However, when the amino acids were present in the medium at $\frac{1}{4}$ or $\frac{1}{8}$ the concentration, development to the blastocyst stage and blastocyst hatching were significantly reduced.

Interestingly, reducing the essential amino acid concentration by $\frac{1}{2}$ resulted in blastocysts with significantly increased blastocyst cell numbers, ICM, and percentage ICM cell numbers compared with the medium containing Eagle's concentration of essential amino acids (Table III). There was no difference in blastocyst cell numbers between embryos cultured with $\frac{1}{4}$ essential amino acids and those with the full concentration of Eagle's essential amino acids (Table III). However, reducing the essential amino acid concentration to $\frac{1}{4}$ significantly reduced the percentage of ICM cells in the blastocysts (Table III). Blastocyst cell number, ICM, and TE and percentage

Table II. Effect of the Concentration of Essential Amino Acids on Embryo Development in Culture

Essential amino acid dilution	N	Blastocyst development (%)	Hatching blastocyst/embryo cultured (%)	Hatching blastocyst/blastocyst development (%)
1:1	129	79.8 ^a	32.6 ^a	40.8 ^a
1:2	134	84.3 ^a	42.5 ^b	50.4 ^b
1:4	130	67.9 ^b	23.1 ^c	34.1 ^a
1:8	126	61.1 ^b	22.2 ^c	36.8 ^a
Nonessential amino acids	125	60.0 ^b	14.4 ^c	24.0 ^c

Note. Different superscript letters within a column indicate significantly different values ($P < .05$).

Table III. Effect of the Concentration of Essential Amino Acids on Blastocyst Cell Number and Differentiation

Essential amino acid dilution	Total cell number (Mean $\pm$ SEM)	ICM cell number (Mean $\pm$ SEM)	TE cell number (Mean $\pm$ SEM)	ICM/total cell number (%) (Mean $\pm$ SEM)
1:1	96.4 $\pm$ 4.0 ^a	21.7 $\pm$ 1.1 ^a	75.9 $\pm$ 3.6 ^a	22.4 $\pm$ 0.8 ^a
1:2	110.3 $\pm$ 3.7 ^b	26.8 $\pm$ 0.9 ^b	82.9 $\pm$ 3.5 ^a	24.8 $\pm$ 0.6 ^b
1:4	89.6 $\pm$ 4.1 ^a	17.2 $\pm$ 3.6 ^a	72.4 $\pm$ 3.7 ^a	19.7 $\pm$ 0.7 ^c
1:8	73.8 $\pm$ 3.4 ^c	13.6 $\pm$ 0.8 ^c	61.2 $\pm$ 2.8 ^b	18.1 $\pm$ 0.8 ^c
None	68.5 $\pm$ 2.0 ^c	9.8 $\pm$ 0.5 ^c	59.0 $\pm$ 1.9 ^b	14.2 $\pm$ 0.5 ^d

Note. At least 50 blastocysts counted per treatment. Different superscript letters within a column indicate significantly different values ($P < .05$).

of ICM cells were all reduced when the amino acids were reduced to $1/8$ the original concentration or when there were no essential amino acids in the medium (Table III).

Effect of Essential Amino Acids on Embryo Viability

The effect of reducing the essential amino acid concentration to $1/2$ or $1/4$ for embryo culture on subsequent viability was assessed. Reducing the concentration of essential amino acids to $1/2$ did not affect either implantation rates or fetal development of cultured blastocysts compared with the control (Table IV). However, decreasing the essential amino acids to $1/4$ of the control resulted in a significant decrease in fetal development and weight after transfer compared with both the control and embryos cultured with $1/2$ concentration of essential amino acids (Table IV).

Ammonium Production in the Medium

Medium G2.2 with differing concentrations of essential amino acids was incubated at 37°C with and without embryos for 48 h and ammonium production assessed. Ammonium production in all media increased from 0 h to 24 h and 48 h (Fig. 1). At each time point, the ammonium concentration was significantly higher when embryos were cultured in the drops of medium (Fig. 1). Medium with $1/2$ essential amino acids had significantly lower levels of ammonium at both 24 and 48 h of culture compared with the control

medium ($P < .05$; Fig. 1). Ammonium levels were further reduced when the essential amino acids were reduced to $1/4$ of the control concentrations (Fig. 1). There was only a very low level of ammonium production in medium devoid of essential amino acids due to the breakdown of the nonessential amino acids.

Ammonium Production by Embryos

Ammonium production was equivalent for blastocysts grown in either the control medium or medium containing $1/2$ concentration of essential amino acids (Fig. 2). Ammonium production by blastocysts cultured in medium containing $1/4$ the concentration of essential amino acids was significantly less than blastocysts incubated in the presence of Eagle's concentrations of essential amino acids (Fig. 2).

DISCUSSION

Reducing the concentration of Eagle's essential amino acids in the culture medium to $1/2$ significantly increased development of the postcompaction stage mouse embryo. Reducing the essential amino acid concentration by $1/2$ increased blastocyst development, blastocyst cell numbers, and ICM cell numbers. Further decreasing the concentration of essential amino acids in the medium to $1/4$ or $1/8$ reduced blastocyst development and ICM cell numbers. Viability was also decreased when the essential amino acids were reduced to $1/4$ of the original concentration.

Table IV. Effect of the Concentration of Essential Amino Acids on Embryo Viability

Essential dilution	N	Implantation (%)	Fetal development (%)	Fetal development implantation (%)	Fetal weight (mg) (Mean $\pm$ SEM)
1:1	60	58.3 ^a	35.0 ^a	60.0	298.7 $\pm$ 7.0 ^a
1:2	60	66.7 ^a	41.7 ^a	62.5	302.0 $\pm$ 4.0 ^a
1:4	60	25.0 ^b	16.7 ^b	66.7	276 $\pm$ 12.9 ^b

Note. Different superscript letters within a column are significantly different values ($P < .05$).

Fig. 1. Effect of essential amino concentration on ammonium production in the medium: (a) no embryos; (b) with 10 embryos. + represents Eagle's essential amino acids; ○ represents $\frac{1}{2}$ Eagle's essential amino acids; ▲ represents $\frac{1}{4}$ Eagle's essential amino acids. $n = 10$ readings per treatment.

Amino acid requirements by the mouse embryo have been shown to differ between the cleavage stage embryo and the postcompaction stage embryo. Initially, the amino acids classified by Eagle (10) as nonessential stimulate development of the cleavage stage embryo (7,11). Postcompaction, the nonessential amino acids stimulate blastocyst expansion and hatching, whereas the essential amino acids stimulate the inner cell mass (7,11). The presence of Eagle's

essential amino acids during the development of the postcompaction stage embryo also resulted in higher rates of fetal development after transfer (7). However, the concentration of the essential amino acids in culture media for previous studies was significantly higher than the embryo would be exposed to in vivo (12). It has recently been demonstrated that development of the bovine embryo in culture is inversely related to the concentration of essential amino acids in

Fig. 2. Effect of essential amino acid concentration on ammonium production by blastocysts. $n = 20$ embryos per treatment. *Significantly different, $P < 0.05$.

the medium (14). For the postcompaction bovine embryo, reducing the concentration of essential amino acids to $\frac{1}{2}$ increased blastocyst development. Further reducing the concentration of essential amino acids to $\frac{1}{4}$ resulted in highest rates of blastocyst development (14). There appears to be some differences in the effect of essential amino acids on the development of the mouse and cow blastocyst. In contrast to cow embryos, halving the essential amino acid concentration in the medium did not affect the number of CF1 mouse blastocysts produced in culture. However, reducing the essential amino acid concentration to $\frac{1}{2}$ increased both blastocyst hatching rates and blastocyst cell numbers. Although further decreasing the essential amino acid concentration to $\frac{1}{4}$ did not affect blastocyst development or total cell numbers, development of the ICM was reduced. Furthermore, blastocysts cultured with $\frac{1}{4}$ essential amino acid concentration had significantly reduced viability. This study confirmed previous studies that the presence of the essential amino acids in the culture medium for the postcompaction stage mouse embryo stimulates inner cell mass development (7). Therefore, to maintain the viability of the mouse embryo in culture it is important to maintain the essential amino acid concentration to at least $\frac{1}{2}$ of Eagle's concentrations (Table I) following compaction to ensure normal development of the inner cell mass. It is not certain if this is also the case for the cow blastocyst, because the effect of decreasing the concentration of essential amino acids on the development of the inner cell mass was not assessed (14).

An explanation for the increased development observed when embryos were cultured with $\frac{1}{2}$ concentration of Eagle's essential amino acids is that reducing the concentration of amino acids in the medium decreases the amount of ammonium produced. Amino acids spontaneously break down to ammonium when incubated at 37°C (1). In addition, ammonium is a byproduct of amino acid metabolism (1). The levels of ammonium produced in the culture medium containing all Eagle's amino acids after incubation for only 48 h is inhibitory to embryo development (1). In this study, production of ammonium in the medium decreased significantly with reducing concentrations of essential amino acids in the medium. Interestingly, the levels of ammonium production in this study were lower than that reported previously for media containing Eagle's nonessential and essential amino acids and glutamine (1). The difference between these studies is that a more stable derivative of glutamine, alanylglutamine was used in this study. The increased stability of alanylglutamine results in the production of significantly less ammonium when incubated at 37°C .

An alternative explanation for the increased development observed when the concentration of essential amino acids was reduced by $\frac{1}{2}$ is that decreasing the concentration results in a decrease in the levels of an inhibitory amino acid. It has previously been shown that reducing the concentration of amino acids by $\frac{1}{10}$ th can convert an amino acid from being inhibitory to hamster embryos in culture, to actually stimulating development (20).

Interestingly, the ammonium production by blastocysts incubated in the presence of either Eagle's concentrations of essential amino acids or $\frac{1}{2}$ the concentration was similar. However, further reducing the concentration resulted in a decrease in ammonium production. It is plausible that when the concentration of essential amino acids was reduced to $\frac{1}{4}$, the rate of amino acid transamination was limited by the concentration of the amino acids present. In contrast, when blastocysts were incubated with the amino acids present at either Eagle's concentrations or $\frac{1}{2}$ the concentration, amino acid metabolism was not limited by presence of the amino acids.

In conclusion, reducing the concentration of essential amino acids in the culture medium resulted in the medium being more stable and increased development of the mouse blastocyst in culture. Reducing the essential amino acid concentration below $\frac{1}{2}$ had a negative effect on ICM development and subsequent developmental competence posttransfer. It would therefore seem prudent to add amino acids to the culture medium at a concentration of $\frac{1}{2}$ of that described by Eagle (1959).

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