ANIMAL EXPERIMENTATION

Nonessential Amino Acids and Glutamine Decrease the Time of the First Three Cleavage Divisions and Increase Compaction of Mouse Zygotes In Vitro

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Purpose: The objective of this study was to determine the effect of supplementing embryo culture media with amino acids on the duration of the first three cell cycles of mouse zygotes in vitro.

Methods: Zygotes were cultured in the presence of different groups of amino acids and cleavage assessed every 30 min.

Results: Culture of zygotes with Eagle's nonessential amino acids and glutamine significantly reduced the time of cleavage divisions to the eight-cell stage compared to culture without amino acids. Beneficial effects of amino acids were found to be cumulative over time. Nonessential amino acids and glutamine also increased the percentage of eight-cells that compacted after 57 hr of culture compared to embryos in medium devoid of amino acids.

Conclusions: The present data suggest that media for the development of cleavage-stage embryos, such as in clinical IVF, should be supplemented with Eagle's nonessential amino acids and glutamine.

KEY WORDS: cell cycle; cleavage rate; culture; preimplantation embryo.

INTRODUCTION

Fluid of the female reproductive tract is characterized by high levels of free amino acids (1–3). The amino

acids at the highest concentrations are glycine, taurine, alanine, aspartate, glutamate, and glutamine. With the exception of taurine and glutamine, these amino acids are present in Eagle's group of nonessential amino acids (4). The addition of Eagle's nonessential amino acids and glutamine to embryo culture medium significantly increases the development of mouse zygotes to the blastocyst stage after 3 days of culture (5). Importantly, nonessential amino acids and glutamine also alleviate the two-cell block in CF1 mouse embryo development (6). The removal of glutamine from this group reduced the stimulatory effects of amino acids on development. Furthermore, the addition of nonessential amino acids and glutamine to embryo culture medium results in an increase in both implantations and subsequent fetal development when embryos are transferred after 48 hr of culture from the zygote, at the six- to eight-cell stage (7). In contrast, the addition of those amino acids at the lowest levels in the tract, those present in Eagle's essential amino acids without glutamine, to embryo culture medium results in a significant reduction in resultant blastocyst cell numbers after 4 days of culture. Furthermore, such amino acids confer no benefit to the precompacted embryo after transfer (5,7).

It has been demonstrated that hamster embryos that undergo more rapid cleavage in culture have a higher developmental potential after transfer (8,9). It was therefore the aim of this study to determine the effect of specific groups of amino acids on the timing of the first three cell cycles of mouse embryos in culture and to see whether this correlated with their known effects on development and viability. Nonessential amino

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acids and glutamine were chosen, as they appear to convey the maximum benefit to the embryo, while essential amino acids without glutamine were chosen, as this group does not confer any benefit to the cleavage stage embryo. All 20 amino acids were also examined to determine the effectiveness or otherwise of the two groups of amino acids together.

MATERIALS AND METHODS

Medium Composition

The basic medium used in this study was mMTF (5), which had the following composition—NaCl, 98.4 mM; KCl, 4.79 mM; MgSO₄, 1.19 mM; KH₂PO₄, 1.19 mM; CaCl₂, 1.71 mM; NaHCO₃, 25.0 mM; sodium pyruvate, 0.37 mM; DL-sodium lactate, 4.79 mM; glucose, 3.4 mM; phenol red, 0.10 g/L; penicillin, 0.06 g/L; and streptomycin, 0.05 g/L—and was supplemented with 4 g/L BSA. Amino acids were added to medium mMTF at the concentrations found in Eagle's medium with the exception of glutamine, which was used at a concentration of 1 mM (Table I). Media supplemented with amino acids had their sodium chloride composition lowered to maintain osmolarity at 260 mosmol. Media for embryo collection and manipulation had 20.0 mM NaHCO₃ replaced with 20.0 mM HEPES at pH 7.4.

All salts were Analar grade (BDH, Poole, Dorest, UK) and carbohydrates, amino acids, antibiotics, hyaluronidase, and HEPES were cell culture grade (Sigma Chemical Co., St Louis, MO). BSA was purchased

from Bayer Diagnostics (Miles Pentex Crystalline, lot 97, Kanakee, IL).

Four culture treatments were used in the study: mMTF (control), mMTF supplemented with nonessential amino acids and 1 mM glutamine (NEGLN), mMTF supplemented with essential amino acids without glutamine (ESS), and mMTF supplemented with all 20 of Eagle's amino acids (20AA).

Embryo Collection and Culture

Embryos were collected from F₁ (C57/BL6 × CBA/Ca) hybrid females superovulated with 5 IU pregnant mares' serum gonadotropin (Folligon; Intervet, Lypard, Victoria, Australia), followed 48 hr later by 5 IU of human chorionic gonadotropin (hCG; Chorulon; Intervet). Females were placed with males of the same strain and mating was assessed the following morning by the presence of a vaginal plug. Zygotes were collected at 21 hr post-hCG and denuded by a 1-min incubation with hyaluronidase (1 g/L). Zygotes were washed twice in medium mMTF before allocation to one of the four culture treatments. For each of the three cell cycles investigated a separate dish of embryos was used. Embryos from each female were distributed evenly among the four culture treatments and the three cell cycles (12 embryos). Embryos designated for each cell cycle analysis were examined every 30 min around the time of cleavage. As a control for the frequency at which an individual dish was examined, a parallel dish was set up which was removed from the incubator only twice during each cell cycle. Therefore a total of 24 embryos was required from each mouse. All embryos were cultured in groups of 10 in 20-μl drops of medium at a gas phase of 5% CO₂ in air in modular incubator chambers within an incubator. To alleviate the stress of constant opening of the incubator, embryos that were assessed every 30 min were housed in a separate incubator. At the end of each cell cycle the set of dishes that had been examined at 30-min intervals was discarded when all of the embryos had completed the cell cycle of interest. A different set of dishes was then used for analysis of each subsequent cell cycle. At least 30 embryos were cultured per treatment and cell cycle (a total of 720 embryos for the study).

Embryo Morphology

Embryo morphology was assessed using phase-contrast microscopy with Nomaski Optics at 37°C. Age of embryos is expressed as hours post hCG. Time in culture can be calculated by subtracting 21 hr.

Table I. Concentrations of Amino Acids Used to Supplement Culture Media^a

Nonessential amino acid	Concentration (mM)	Essential amino acid	Concentration (mM)
Alanine	0.1	Arginine	0.6
Asparagine	0.1	Cystine	0.1
Aspartate	0.1	Histidine	0.2
Glutamate	0.1	Isoleucine	0.4
Glycine	0.1	Leucine	0.4
Proline	0.1	Lysine	0.4
Serine	0.1	Methionine	0.1
		Phenylalanine	0.2
		Threonine	0.4
		Tryptophan	0.5
		Tyrosine	0.2
		Valine	0.4

^a Glutamine, an essential amino acid as defined by Eagle, was used at 1.0 mM.

Statistical Analysis

Data for cleavage rates were assessed using analysis of variance. Differences between treatments was determined using the Bonferroni procedure for multiple comparisons. Data for rates of compaction were assessed using linear logistic regression assuming the error distribution to be binomial. Differences between treatments were assessed using the LRS statistic, which is approximately chi-square distributed (10). Comparisons of embryo cell numbers of dishes examined every 30 min compared to dishes examined every 6 hr were assessed using Student's *t* test. Values are expressed as mean $\pm$ SE.

RESULTS

There were no significant differences in the mean cell cycle times in the dishes examined at either 30 min or 6 hr intervals. Furthermore, there was no difference in the results between the two replicates performed, and therefore the data from both replicates were pooled.

Cleavage from the Zygote to the Two-Cell Stage

Zygotes cultured in medium mMTF cleaved to the two-cell stage over a 5.5 hr period (Fig. 1a). The mean time for cleavage to the two-cell stage in mMTF was 33.0 ± 0.3 hr post-hCG. Culture with amino acids did not significantly effect the timing of cleavage of zygotes to the two-cell stage (nonessential amino acids and glutamine, 32.5 ± 0.2 ; essential amino acids without glutamine, 33.1 ± 0.2 ; and all 20 amino acids, 32.8 ± 0.2 ; Fig. 1a).

Cleavage from the Two-Cell to the Four-Cell Stage

The mean cleavage time of two-cell embryos to the four-cell stage was significantly reduced by culture with either nonessential amino acids and glutamine (56.5 ± 0.3 hr post-hCG; $P < 0.01$) or all 20 amino acids (57.5 ± 0.3 hr post-hCG; $P < 0.05$) compared to embryos cultured in medium mMTF (59.0 ± 0.3 hr post-hCG; Fig. 1b). Nonessential amino acids and glutamine also reduced the cleavage time compared to culture with essential amino acids without glutamine ($P < 0.01$). Essential amino acids without glutamine had no effect on the timing of the second cleavage division (58.3 ± 0.3 hr post-hCG; Fig. 1b).

Fig. 1. Effect of amino acids on cleavage rates from the zygote to the eight-cell stage. (a) Cleavage times from the zygote to the two-cell stage. (b) Cleavage times from the two- to the four-cell stage. (c) Cleavage times from the four- to the eight-cell stage. n = at least 30 embryos per treatment group. Notches represent the interquartile range, therefore including 50% of the data. Whiskers represent 5 and 95% quartiles (11). The line across the box represents the mean. NEGLN, mMTF supplemented with nonessential amino acids and glutamine. ESS, mMTF supplemented with essential amino acids without glutamine. 20AA, mMTF supplemented with all 20 amino acids. *Significantly different from mMTF ($P < 0.05$). **Significantly different from mMTF ($P < 0.01$). ^aLike pairs significantly different ($P < 0.01$). ^bLike pairs significantly different ($P < 0.05$).

Cleavage from the Four-Cell to the Eight-Cell Stage

A similar pattern of cleavage rates was observed for the third cleavage division. Culture with nonessential amino acids and glutamine significantly decreased the

mean cleavage time from 74.7 ± 0.3 hr post-hCG in mMTF to 70.7 ± 0.2 hr post-hCG ($P < 0.01$; Fig. 1c). Culture with all 20 amino acids (73.2 ± 0.4 hr post-hCG) also significantly reduced the mean cleavage time compared to mMTF ($P < 0.05$). There was a significant decrease in the mean cleavage time between embryos cultured in the nonessential amino acids and glutamine and embryos cultured with all 20 amino acids ($P < 0.05$) and essential amino acids without glutamine ($P < 0.01$). Essential amino acids without glutamine did not alter the cleavage rates from the four-cell stage to the eight-cell stage (73.7 ± 0.4 hr post-hCG) compared to the control (Fig. 1c).

The observed reduction in cleavage time to the eight-cell stage by embryos cultured in the presence of nonessential amino acids and glutamine was found to be cumulative, and not specific to a single cleavage division. Cleavage times were reduced with the nonessential amino acids and glutamine from the two- to the four-cell stages and from the four- to the eight-cell stages compared to culture with mMTF (Table II). However, the reduced mean time for cleavage to the eight-cell stage of embryos cultured with all 20 amino acids was due solely to a reduction in the time of the second cell cycle. All 20 amino acids did not effect the cleavage rate from the four-cell to the eight-cell stage (Table II).

Frequency of Compaction

By the end of the experimental period, at 78 hr post-hCG (57 hr of culture), there were significant differences in the proportion of eight-cell embryos that had compacted (Fig. 2). Of the eight-cell embryos cultured in medium mMTF, only 6% had compacted. Similarly only 8% of embryos cultured in the presence of essential amino acids without glutamine had com-

Fig. 2. Effect of amino acids in the medium on the compaction of eight-cell embryos after 78 hr post-hCG (57 hr of culture). $n =$ at least 30 embryos per treatment. Values are mean $\pm$ SE. NEGLN, mMTF supplemented with nonessential amino acids and glutamine. ESS, mMTF supplemented with essential amino acids without glutamine. 20AA, mMTF supplemented with all 20 amino acids. **Significantly different from mMTF and ESS ($P < 0.01$). ^aLike pairs significantly different ($P < 0.05$).

pacted 78 hr post-hCG (Fig. 2). Significantly more eight-cell embryos cultured in either nonessential amino acids and glutamine (76%; $P < 0.01$) or all 20 amino acids (48%; $P < 0.01$) were compacted compared to no amino acids (mMTF) or essential amino acids without glutamine. Furthermore, significantly more eight-cell embryos were compacted after culture with nonessential amino acids and glutamine compared to all 20 amino acids ($P < 0.05$) (Fig. 2).

DISCUSSION

This study has demonstrated that culture with Eagle's nonessential amino acids and glutamine significantly reduces the cleavage time of zygotes to the eight-cell stage. This reduction in cleavage time was found to be due to a reduction in mean cleavage time from the two- to the eight-cell stage. Furthermore,

Table II. Effect of Amino Acids on the Timing of Each Individual Cleavage Division from the Zygote to the Eight-Cell Stage

Cleavage division	Cell cycle length (hr)			
	mMTF	NEGLN	ESS	20AA
Zygote to 2-cell ^a	12.1 $\pm$ 0.3	11.5 $\pm$ 0.2	12.1 $\pm$ 0.2	11.8 $\pm$ 0.2
2-cell to 4-cell	25.9 $\pm$ 0.3	24.1 $\pm$ 0.3*	25.6 $\pm$ 0.3	24.8 $\pm$ 0.2*
4-cell to 8-cell	15.9 $\pm$ 0.4	14.0 $\pm$ 0.2**	15.6 $\pm$ 0.5	15.7 $\pm$ 0.3

^a Time of cleavage taken from time of collection of zygotes (21 hr post-hCG). $n =$ at least 30 embryos per treatment. Values are mean $\pm$ SE. NEGLN, mMTF supplemented with nonessential amino acids and glutamine. ESS, mMTF supplemented with essential amino acids without glutamine. 20AA, mMTF supplemented with all 20 amino acids.

* Significantly different from mMTF ($P < 0.05$).

** Significantly different from mMTF ($P < 0.01$).

culture with nonessential amino acids and glutamine also significantly increased the percentage of eight-cell embryos that compacted after 78 hr post-hCG (57 hr of culture). Culture with Eagle's essential amino acids without glutamine did not affect the cleavage rates of zygotes to the eight-cell stage or the percentage of eight-cell embryos that compacted after 57 hr of culture. Culture with all 20 amino acids significantly reduced the overall cleavage times of the zygote to the eight-cell stage, however, the stimulatory effect was found to be due to reducing the cleavage time from the two- to the four-cell stage. Culture with all 20 amino acids did not alter cleavage rates from the four- to the eight-cell stage. All 20 amino acids increased the proportion of eight-cell embryos compacting compared to embryos cultured without amino acids. This, however, was significantly less than that observed for embryos cultured with nonessential amino acids and glutamine.

The reported benefit of nonessential amino acids and glutamine is consistent with published work on their stimulatory effects on the mouse embryo (5-7). In contrast to the beneficial effects of nonessential amino acids and glutamine, Eagle's essential amino acids without glutamine conferred no observed benefit to the cleavage stage embryo. It has been reported previously that culture of zygotes with essential amino acids without glutamine reduced the subsequent blastocyst cell number after 96 hr of culture (5). Furthermore, although culture of zygotes with essential amino acids without glutamine for 48 hr prior to transfer did not affect pregnancy rates compared to embryos cultured without amino acids, resultant fetuses were significantly lighter (7).

While all 20 amino acids stimulated cleavage rates to the four-cell stage, their effect on cleavage time was reduced compared to that of nonessential amino acids, at all stages of development. It is possible that this reduced effect of all 20 amino acids is due to the presence of essential amino acids. Although the essential amino acids did not have any detrimental effects on their own, it is possible that in the presence of the nonessential amino acids and glutamine (i.e., all 20 amino acids), they counteract the stimulatory effect of the nonessential amino acids and glutamine.

Alternatively, as the stimulatory effect of all 20 amino acids on cleavage rates diminished over time, it is plausible that the loss in stimulation was due to the buildup of ammonium in the medium. Culture medium containing 20 amino acids incubated for 48 hr in the presence of embryos has been reported to have an ammonium concentration of 0.239 mM, con-

siderably higher than the concentration present in medium containing only the nonessential amino acids and glutamine, 0.112 mM (5). The presence of this concentration of ammonium in the medium significantly reduces development to the blastocyst stage (5) and also reduces viability after transfer (7).

Nonessential amino acids and glutamine have been demonstrated previously to increase development to the blastocyst stage after just 3 days of culture (5), as well as facilitating development beyond the 2-cell block in CF1 mouse embryos (6) and beyond the 8- to 16-cell block in sheep embryos (12). Furthermore, mouse zygotes cultured with nonessential amino acids and glutamine for 48 hr to the eight-cell stage have an increased fetal development after transfer compared to embryos cultured without amino acids (7). An increased viability of faster cleaving embryos to the eight-cell stage has been reported in the hamster (8,9), indicating that the rate of cleavage to the eight-cell stage is an indicator of subsequent viability. Consistent with these data, those amino acids which increased fetal development in the mouse (nonessential amino acids and glutamine) reduced the time of the first three cell cycles. The latter is an important point for clinical IVF, in which embryos are not transferred at the blastocyst stage but, rather, are routinely cultured for only 2 or 3 days prior to replacement. To this end, it was demonstrated in a pilot trial that a medium designed specifically for the development of the cleavage-stage human embryo, medium G1 (13), increased human embryo development in vitro (14). Medium G1 contains Eagle's nonessential amino acids and glutamine. Culture of pronuclear-stage human embryos for 24 hr in medium G1 increased the percentage of embryos beyond the four-cell stage (79%) compared to embryos, cultured from the same patients, in a medium lacking amino acids (45%) (14). Furthermore, embryos cultured in medium G1 exhibited significantly less fragmentation than embryos cultured in a medium without amino acids (14). It is therefore evident from this and previous studies that nonessential amino acids and glutamine confer benefit on the first three cell cycles of mammalian embryo development in culture and therefore their inclusion in embryo culture media is warranted.

Importantly, the mode of action of this group of amino acids is open to speculation and investigation (15,16). Effects of nonessential amino acids and glutamine on the cleavage-stage embryo include protection against osmotic/ionic stress (17,18), regulators of metabolic function (19), and regulators of intracellular pH (Gardner and Edwards, unpublished observations).

Further studies on the role of amino acids in embryo development are required to further increase our ability to culture viable mammalian embryos.

CONCLUSIONS

Eagle's nonessential amino acids and glutamine reduced the time for the first three cell cycles of mouse zygotes in vitro. In contrast, Eagle's essential amino acids did not affect development, while all 20 amino acids increased development only during the second cell cycle. As embryos are routinely cultured to the four- to eight-cell stage prior to transfer in human IVF, it is proposed that the effectiveness of nonessential amino acids and glutamine on human embryo development and viability be evaluated.

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REFERENCES

1. Fahning ML, Schultz RH, Graham EF: The free amino acid content of uterine fluids and blood serum in the cow. *J Reprod Fertil* 1967;13:229-236
2. Menezo YJR: Amino constituents of tubal and uterine fluids of the estrous ewe: comparison with blood serum and ram seminal fluid. *In* The Biology of Spermatozoa, ESE Hafex, CG Thibault (eds). New York, Basel Press, 1975, pp 174-181
3. Miller JGO, Schultz RM: Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. *Biol Reprod* 1987;36:125-129
4. Eagle H: Amino acid metabolism in mammalian cell cultures. *Science* 1959;130:432-437
5. Gardner DK, Lane M: Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod* 1993;48:377-385
6. Gardner DK, Lane M: Alleviation of the "2-cell-block" and development to the blastocyst of CF1 mouse embryos: Role of amino acids, EDTA and physical parameters. *Hum Reprod* 1996;11:2703-2712
7. Lane M, Gardner DK: Increase in post-implantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *J Reprod Fertil* 1994;102:305-312
8. McKiernan SH, Bavister BD: Timing of development is a critical parameter for predicting successful embryogenesis. *Hum Reprod* 1994;9:2123-2129
9. Gonzales DS, Pinheiro JC, Bavister BD: Prediction of the developmental potential of hamster embryos in vitro by precise timing of the third cell cycle. *J Reprod Fertil* 1995;105:1-8
10. Nelder JA, Wedderburn RWM: Generalized linear models. *J Rev Stat Soc* 1972;135:370-383
11. Kafadar K: Notched box-and-whisker plot. *Enc Stat Sci* 1985;6:367-370
12. Gardner DK, Lane M, Spitzer A, Batt PA: Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: Amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod* 1994;50:390-400
13. Barnes FL, Crombie A, Gardner DK, Kausche A, Lacham-Kaplan O, Suikkari A-M, Tiglias J, Wood C, Trounson A: Blastocyst development and birth after in vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Hum Reprod* 1995;10:3243-3247
14. Gardner DK: Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int* 1994;18:1163-1179
15. Gardner DK, Lane M: Embryo culture systems. *In* Handbook of In Vitro Fertilization. AO Trounson, DK Gardner (eds). Boca Raton; FL, CRC Press, 1993, pp 85-114
16. Bavister BD: Culture of preimplantation embryos: Facts and artifacts. *Hum Reprod Update* 1995;1:91-148
17. Van Winkle LJ, Haghighat N, Campione AL: Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. *J Exp Zool* 1990;253:215-219
18. Lawitts JA, Biggers JD: Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. *Mol Reprod Dev* 1992;31:189-194
19. Gardner DK, Lane M: The 2-cell block in CF1 mouse embryos is associated with an increase in glycolysis and a decrease in tricarboxylic acid (TCA) cycle activity: Alleviation of the 2-cell block is associated with the restoration of in vivo metabolic pathway activities. *Biol Reprod* 1993;49 (Suppl 1):152