Effect of different concentrations of amino acids in human serum and follicular fluid on the development of one-cell mouse embryos *in vitro*

T. Nakazawa^{1*}, K. Ohashi², M. Yamada¹, S. Shinoda¹, F. Saji²,
Y. Murata² and H. Araki¹

¹Research and Development Center, Fuso Pharmaceutical Industries Ltd, 2-3-30, Morinomiya, Joto-ku, Osaka 536, Japan; and ²Department of Obstetrics and Gynecology, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565, Japan

As a model for establishing an optimized medium for human *in vitro* fertilization (IVF), modified human tubal fluid (HTF) media containing amino acids at concentrations found in human serum and follicular fluid were prepared, and the effect of the media on development of random-bred (ICR) and F₁ hybrid (CBF1) mice embryos was studied. The total concentrations of amino acids found in serum and follicular fluid were about one-third to one-half the concentrations present in two conventional media used in human IVF: Ham's F-10 and Eagle's minimal essential medium (MEM). When ICR mouse embryos were cultured in the HTF medium containing 21 amino acids at concentrations found in follicular fluid, the number of embryos developing to morulae at 72 h and to blastocysts at 96 h increased in comparison with those cultured in HTF medium. When HTF containing amino acids at concentrations found in serum was used, only induced morula formation at 72 h was enhanced. The number of hatching blastocysts at 96 h also increased when CBF1 mouse embryos were cultured with HTF supplemented with amino acids at concentrations found in follicular fluid. When ICR mouse embryos were cultured in modified HTF media containing concentrations of amino acids found in Ham's F-10 and MEM that contained higher concentrations of glutamine, embryo development was inhibited. The amount of ammonium produced during incubation for 3 days was significantly less when embryos were cultured in media containing concentrations of amino acids found in follicular fluid compared with when Ham's F-10 or MEM was the culture medium. Ammonium is produced by the breakdown of glutamine in the culture medium during incubation with or without embryos. These results suggest that the concentrations of amino acids found in follicular fluid are more effective and safer for embryo culture than those in other media currently in use.

Introduction

The addition of serum to culture medium, conditioned medium and somatic cell co-culture systems supports the development *in vitro* of embryos from various mammalian species (Eyestone and First, 1989; Myers *et al.*, 1994; Rorie *et al.*, 1994). However, these culture systems are not favoured for human *in vitro* fertilization (IVF) because of their potential contamination with undesirable components such as viruses. To avoid viral infection, it is important to prepare a serum-free medium that provides optimal conditions for embryo development. In conventional human IVF, embryos are cultured in commercially available media such as Ham's F-10, Eagle's MEM and α -MEM, which have been developed for somatic cell culture. These culture media contain components, such as amino acids, vitamins and energy substrates, to enhance the growth of somatic

cells. However, it is not known whether these components are required for embryo development.

When mammalian embryos are cultured in medium containing amino acids, the embryonic cells take up amino acids (Brinster, 1971; Epstein and Smith, 1973; Kaye *et al.*, 1982), and the presence of amino acids enhances embryo development (Kane and Foote, 1970; Kane *et al.*, 1986; Miyoshi *et al.*, 1995). One reason for the requirement for amino acids is that they are substrates for protein synthesis, which occurs to a considerable extent in early embryos (Morgan and Kane, 1993; Lamb and Leese, 1994). However, ammonium produced by embryo metabolism and by the spontaneous breakdown of amino acids in the culture medium inhibits embryo development (Gardner and Lane, 1993). This findings suggests that media for IVF should not contain amino acids at concentrations higher than those needed for embryo development.

In the present study, the effects of supplementation of human tubal fluid (HTF; Quinn *et al.*, 1985) medium with amino acids on the development of mouse embryos *in vitro* were

*Correspondence.

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analysed. Human follicular fluid and serum, as well as two conventional media, Ham's F-10 (Ham, 1963) and Eagle's minimal essential medium (MEM) (Eagle, 1959) which are frequently used as culture media for IVF, were used as amino acid sources. Random-bred ICR and F_1 hybrid CBF1 strains of mice were used. In random-bred mice, embryo development is depressed at the two-cell stage in traditional culture, while F_1 hybrid mouse embryos progress through this block (Dandekar and Glass, 1987). Embryos were cultured in the modified HTF media and embryo development was observed as a function of the concentrations of free amino acids and the production of ammonium.

Materials and Methods

Collection of human follicular fluid and serum

Human follicular fluid and serum were collected from 21 women enrolled in the IVF program at Osaka University Hospital. Informed consent was obtained. Patients received human menopausal gonadotrophin and buserelin acetate. Follicular growth was monitored by ultrasonography. When the leading follicle reached 18 mm in diameter, 10 000 iu human chorionic gonadotrophin (hCG) was administered. Oocytes were recovered transvaginally 36 h after the hCG injection. Venous blood was collected before oocyte recovery. Follicular fluid and blood were centrifuged at 400 *g* for 10 min at 4°C, and the supernatants were stored below -20°C. Individual fluid samples were kept separately until use.

Measurement of free amino acids

Twenty-one samples of follicular fluid and serum were deproteinized by the addition of an equal volume of 5% (w/v) sulfosalicylic acid solution and then centrifuged at 1500 *g* for 10 min at 4°C. The individual concentrations of 21 free amino acids were measured with an amino acid analyser (Hitachi L-2500, Tokyo).

Preparation of culture media

HTF medium containing no amino acids was used as basic medium, and all culture media containing amino acids were prepared by the addition of amino acids to this medium. Four media were prepared containing amino acids at concentrations found in follicular fluid, serum, Ham's F-10 medium and MEM. All media were supplemented with 0.5% (w/v) BSA (Sigma, St Louis, MO) and were passed through a 0.22 micrometer filter (Mirex GV, Millipore, Bedford, MA).

Collection and culture of mouse embryos

Random-bred ICR and F_1 hybrid CBF1 mice (obtained from Japan Clea, Tokyo, and Japan SLC, Shizuoka, respectively) were given an i.p. injection of 5 iu pregnant mares serum gonadotrophin (PMSG, Teikoku Zoki, Tokyo) followed by 5 iu hCG (Teikoku Zoki) 48 h after PMSG injection. Female mice were mated with male mice of the same strain and killed 18 h after

the hCG injection to recover one-cell embryos from the oviducts. The embryos were treated with 0.1% (w/v) hyaluronidase (Sigma) in HTF medium containing 0.5% (w/v) BSA (HTF/0.5%BSA) to remove cumulus cells, and then rinsed with HTF/0.5% BSA. Embryos from 5–7 females were pooled and placed randomly in groups of 10–20 into 100 μ l droplets of a pre-equilibrated test medium on a 65 mm diameter Petri dish, and incubated for 72 h and 96 h under 5% CO₂ in air at 37°C.

Embryo development was observed with a phase-contrast microscope after incubation period.

Determination of ammonium and free amino acids during incubation

Each medium was pre-equilibrated overnight in 100 μ l droplets under a layer of paraffin oil and then incubated with and without ten ICR embryos for 3 days at 37°C in 5% CO₂ in air. Aliquots (50 μ l) of each medium were processed for determination of ammonium after 0, 24 and 72 h of incubation. Determination of ammonium was performed by enzymatic analysis (Ammoniak, Boehringer Mannheim, Mannheim). The remaining 30 μ l of each sample was processed simultaneously for determination of free amino acids as described above.

Statistical analyses

The results of the determination of free amino acids were analysed by the non-paired Student's *t* test. The percentage of embryos developed to various stages was calculated for each experimental group, and the data were analysed by a one-way analysis of variance (ANOVA). When the ANOVA test showed significant differences ($P < 0.05$), differences between individual treatments were assessed by the multiple comparisons test according to Tukey ($P < 0.05$).

Results

The concentrations of free amino acids in human follicular fluid, serum, Ham's F-10 and MEM media are listed (Table 1). Eleven amino acids were present at significantly higher concentrations in serum than in follicular fluid. Ham's F-10 contains all amino acids except taurine, whereas MEM lacks nine amino acids. These conventional media contained over 5–11 times the concentration of glutamine found in serum and follicular fluid. The total concentration of amino acids was also higher (1.5 to 3.1 times) in conventional media than in serum and follicular fluid.

One-cell embryos were cultured in HTF medium and two modified HTF media containing amino acids at concentrations found in follicular fluid (HTF(FF)) and serum (HTF(serum)). Embryo development was observed at 72 h and 96 h after incubation (Table 2). In the two modified HTF media, a greater percentage of the ICR mouse embryos cultured had developed to the morula stage after 72 h of culture than those cultured in HTF medium. At 96 h of incubation, the percentages of total blastocysts in HTF(FF) were also higher than those in HTF medium. In contrast, blastocyst formation was not increased in HTF(serum). CBF1 mouse embryos developed well in all three

Table 1. Amino acid concentrations (mean $\pm$ SEM) in follicular fluid (FF), serum, Ham's F-10 and Eagle's MEM

Amino acids ($\mu\text{mol l}^{-1}$)	FF	Serum	Ham's F-10	MEM
Eagle's essential amino acids				
Arg	53.2 $\pm$ 3.5 ^a	75.6 $\pm$ 6.1	1000	600
Cys-Cys	5.6 $\pm$ 1.2	6.0 $\pm$ 1.7	*	100
Gln	178.8 $\pm$ 8.5	200.4 $\pm$ 7.1	1000	2000
His	77.5 $\pm$ 2.3 ^a	90.1 $\pm$ 2.3	100	200
Ile	34.1 $\pm$ 1.4 ^a	58.2 $\pm$ 2.2	20	400
Leu	60.1 $\pm$ 2.5 ^a	107.9 $\pm$ 4.2	100	400
Lys	133.7 $\pm$ 5.8 ^a	164.5 $\pm$ 5.7	160	400
Met	14.3 $\pm$ 0.8 ^a	21.0 $\pm$ 0.6	30	100
Phe	41.7 $\pm$ 1.3 ^a	56.1 $\pm$ 2.6	30	200
Thr	124.0 $\pm$ 6.2 ^b	142.1 $\pm$ 6.3	30	400
Trp	33.0 $\pm$ 2.2	38.5 $\pm$ 2.5	3	50
Tyr	42.5 $\pm$ 2.1	48.0 $\pm$ 2.4	10	200
Val	141.4 $\pm$ 6.0	186.6 $\pm$ 7.4	30	400
Eagle's non-essential amino acids				
Ala	298.8 $\pm$ 17.2	317.6 $\pm$ 18.0	100	*
Asn	64.8 $\pm$ 8.9	77.2 $\pm$ 10.5	100	*
Asp	6.4 $\pm$ 0.4 ^a	21.6 $\pm$ 2.1	100	*
Cys	ND	ND	150	*
Glu	92.6 $\pm$ 4.7	89.7 $\pm$ 9.0	100	*
Gly	154.3 $\pm$ 11.2	182.0 $\pm$ 10.6	100	*
Pro	101.4 $\pm$ 4.3	109.0 $\pm$ 5.5	100	*
Ser	73.8 $\pm$ 4.1 ^a	126.6 $\pm$ 5.5	100	*
Tau	31.3 $\pm$ 3.8 ^a	123.7 $\pm$ 12.5	*	*
Total	1763.4 $\pm$ 95.2 ^a	2242.4 $\pm$ 69.8	3363	5450

ND: not determined, *: not included.

^{a,b}Significantly different from serum amino acids at $P < 0.01$, $P < 0.05$ respectively (non-paired Student's *t* test). Arg: Arginine, Cys-Cys: Cystine, Gln: Glutamine, His: Histidine, Ile: Isoleucine, Leu: Leucine, Lys: Lysine, Met: Methionine, Phe: Phenylalanine, Thr: Threonine, Trp: Tryptophan, Tyr: Tyrosine, Val: Valine, Ala: Alanine, Asn: Asparagine, Asp: Aspartic acid, Cys: Cysteine, Glu: Glutamic acid, Gly: Glycine, Pro: Proline, Ser: Serine, Tau: Taurine.

media, and hatching blastocysts were observed after 96 h of incubation in 27.4% (HTF), 56.2% (HTF(FF)) and 53.2% (HTF-serum)) of embryos.

The results of culturing ICR mouse embryos in HTF medium and three modified HTF media containing amino acids at concentrations found in follicular fluid (HTF(FF)), Ham's F-10 (HTF(F-10)) and MEM (HTF(MEM)) are shown (Table 3). The percentages of morulae at 72 h and blastocysts at 96 h in HTF(FF) were significantly higher than those in HTF medium and HTF(MEM). Use of HTF(F-10) and HTF(MEM) did not improve embryo development, and HTF(MEM) inhibited development to the expanded blastocyst stage at 96 h when compared with culture in HTF(FF).

The concentrations of ammonium in media during incubation are shown (Fig. 1a). Concentrations of ammonium in HTF were below $5 \mu\text{g ml}^{-1}$ throughout the 3 days of incubation. Significantly higher concentrations of ammonium were detected in HTF(MEM) than in HTF at the start of the incubation. The concentration of ammonium in the media increased linearly with incubation time in the three media containing amino acids. High concentrations of ammonium (69.1 and $34.2 \mu\text{g ml}^{-1}$) were observed in HTF(MEM) and HTF(F-10) media, respectively, after 3 days of incubation. However, the concentration of ammonium ($7.6 \mu\text{g ml}^{-1}$) was

low in HTF(FF) medium during the 3 days of incubation. Ammonium concentrations were similar when ten embryos were cultured in HTF ($2.9 \mu\text{g ml}^{-1}$), HTF(FF) ($10.1 \mu\text{g ml}^{-1}$), HTF(MEM) ($75.3 \mu\text{g ml}^{-1}$) and HTF(F-10) ($38.4 \mu\text{g ml}^{-1}$) media.

To determine the source of ammonium in the media, the breakdown of each amino acid in the culture media was measured. There was no significant reduction of the concentrations of the amino acids, with the exception of glutamine. The breakdown of glutamine during incubation is shown (Fig. 1b). The reduction of glutamine concentrations paralleled the production of ammonium in each media. The reduction in glutamine concentration in HTF(FF) during incubation was minimal, when compared with that in HTF(MEM) or HTF(F-10), and the presence of embryos made no difference.

Discussion

Amino acids, vitamins and energy sources have been used to supplement culture media in an attempt to create optimal conditions for embryo development *in vitro*. Several reports have shown that supplementation with amino acids can enhance embryo development (Kane and Foote, 1970; Kane

Table 2. Effects of amino acids present in follicular fluid (FF) and serum and added to human tubal fluid (HTF) medium on development of ICR and CBF1 one-cell mouse embryos

Medium	Number of embryos	Percentage of one-cell embryos developing to					
		< Morula	Morula	Blastocyst	Expanded blastocyst	Hatching blastocyst	Total blastocysts
ICR mouse embryos after 72 h of culture							
HTF	81	54.4 ^a	45.6 ^a	0	0	0	0
HTF(FF)	80	19.6 ^b	80.4 ^b	0	0	0	0
HTF(serum)	77	25.6 ^b	72.6 ^b	1.6	0	0	1.6
ICR mouse embryos after 96 h of culture							
HTF	81	54.4 ^a	18.2 ^a	7.0	20.2	0	27.4 ^a
HTF(FF)	80	19.6 ^b	26.4 ^{a,b}	9.0	39.0	6.2	53.8 ^b
HTF(serum)	77	25.6 ^b	32.0 ^b	10.0	26.2	6.2	42.2 ^{a,b}
CBF1 mouse embryos after 72 h of culture							
HTF	67	13.2	72.8	14.0	0	0	14.0
HTF(FF)	69	8.4	60.8	30.8	0	0	30.8
HTF(serum)	68	12.0	63.4	25.0	0	0	25.0
CBF1 mouse embryos after 96 h of culture							
HTF	67	13.2	0	9.0	50.6	27.4 ^a	87.0
HTF(FF)	69	8.4	2.0	3.4	30.0	56.2 ^b	89.6
HTF(serum)	68	12.0	6.0	0	29.0	53.2 ^{a,b}	82.0

Values are expressed as mean $\pm$ SEM. Values with different superscripts within each column are significantly different (ANOVA, Tukey, $P < 0.05$). Experiments were replicated five times.

Table 3. Effects of amino acids in three culture media on the development of ICR one-cell mouse embryos

Medium	Number of embryos	Percentage of one-cell embryos developing to					
		< Morula	Morula	Blastocyst	Expanded blastocyst	Hatching blastocyst	Total blastocysts
Embryos after 72 h of culture							
HTF	78	50.3 ^a	49.7 ^a	0	0	0	0
HTF(FF)	75	19.3 ^b	80.7 ^b	0	0	0	0
HTF(F-10)	72	35.0 ^{a,b}	65.0 ^{a,b}	0	0	0	0
HTF(MEM)	69	51.0 ^a	49.0 ^a	0	0	0	0
Embryos after 96 h of culture							
HTF	78	50.3 ^a	21.5	5.5	22.5 ^a	0	28.0 ^a
HTF(FF)	75	19.3 ^b	20.0	0	47.3 ^b	13.3	60.5 ^b
HTF(F-10)	72	35.0 ^{a,b}	23.0	13.3	26.5 ^{a,b}	2.5	39.8 ^{a,b}
HTF(MEM)	69	51.0 ^a	30.5	12.5	6.0 ^a	0	18.5 ^a

Values are expressed as mean $\pm$ SEM. Values with different superscripts within each column are significantly different (ANOVA, Tukey, $P < 0.05$). Experiments were replicated four times. Human tubal fluid (HTF) was supplemented with amino acids corresponding to composition and concentrations found in follicular fluid (FF), Ham's F-10 medium (F-10) and Eagle's minimum essential medium (MEM). F-10: Ham's F-10.

et al., 1986; Miyoshi *et al.*, 1995). However, the optimal concentration and composition of amino acids has remained unclear. To develop a medium for embryo culture *in vitro*, the concentrations of amino acids in serum and follicular fluid were measured. HTF medium is a common medium for human IVF and does not contain amino acids (Quinn *et al.*, 1985). Therefore, HTF medium was used as a basic medium in this study. Two strains of mice, ICR and CBF1, were used to assess the effectiveness of supplementation of HTF with amino acids.

Culture with media supplemented with amino acids at concentrations present in serum and follicular fluid significantly

stimulated morula formation in ICR mouse embryos after 72 h of incubation. Blastocyst formation after 96 h incubation was stimulated in HTF(FF), but not in HTF(serum). Development of mouse embryos is known to arrest at the two-cell stage (First and Barnes, 1989). In the present study, amino acids mainly facilitated development of early stages of embryos in ICR mice. Early mammalian embryogenesis has been reported to depend on the maternal messenger RNA pool and the transition from maternal to embryonic genome occurs at the two-cell stage in mice (Braude *et al.*, 1979). Therefore, it is likely that amino acids are used as substrates in *de novo* protein synthesis in the early

Fig. 1. (a) Production of ammonium and (b) reduction of glutamine concentration in each media during incubation at 37°C, 5% CO₂ in air with or without mouse embryos. Experiments were replicated three or five times. Values with different superscripts within each column were significantly different (ANOVA, Tukey, $P < 0.05$). Each column represents the mean $\pm$ SEM. Media: $\square$, HTF; $\square$ (hatched), HTF (FF); $\square$ (cross-hatched), HTF (F-10); $\blacksquare$, HTF (MEM).

stage of development. Brinster (1971) reported that incorporation of amino acids into proteins is relatively low at the early preimplantation stage but increases in the later stages. Recent studies have suggested that amino acids are important not only for embryo development *in vitro*, but also for embryo viability and subsequent postimplantation fetal development (Mehta and Kiessling, 1990; Lane and Gardner, 1997). CBF1 mouse embryos develop to the blastocyst stage without supplementation of the culture media with amino acid (Quinn, 1995), while the percentage of hatching blastocysts is improved by supplementation of amino acids at concentrations found in follicular fluid. In the present study, when CBF1 mouse embryos were cultured for 120 h without amino acids, most blastocysts hatched (data not shown). These results suggest that amino acids at concentrations present in follicular fluid facilitate the development of CBF1 mouse embryos. As with the ICR mice, HTF(FF) had a greater beneficial effect on the development of CBF1 embryos than did HTF(serum). Therefore, HTF(FF) medium was used in subsequent experiments.

Ham's F-10 and MEM were developed as media for somatic cell culture and have been used in human IVF. However, supplementation of HTF with amino acids at concentrations

used in these conventional media did not improve significantly development of ICR embryos, and the percentages of morulae at 72 h and of blastocysts at 96 h in HTF(MEM) were lower than those in HTF(FF).

The influence of individual supplementation of free amino acids on embryo development *in vitro* has been reported for several animal species. Bavister and Arlotto (1990) reported that the development of hamster embryos is stimulated by glycine, cysteine and lysine. Moore and Bondioli (1993) found that glycine and alanine enhanced the development of cattle embryos. The beneficial effect of glutamine has also been reported on the development of hamster (Carney and Bavister, 1987), cattle (Rieger *et al.*, 1992), and mouse (Chatot *et al.*, 1990) embryos. In contrast, the present study was designed to assess the effect of a mixture of amino acids at physiological concentrations on embryo development.

Previous studies have examined the effects of a mixture of amino acids on embryo development using commercially available supplements, referred to as Eagle's essential and non-essential amino acids. The addition of Eagle's non-essential amino acids to media is known to be beneficial for embryo development (Liu and Foote, 1995; Gardner and Lane, 1996). However, the present study found that Eagle's essential amino acids, which are identical to MEM-amino acids, are not beneficial for the development of mouse embryos (Gardner and Lane, 1993). Similar findings have been obtained for bovine (Liu and Foote, 1995) and hamster (Bavister and Arlotto, 1990) embryos. In contrast, the combination of non-essential amino acids with essential amino acids at the proportion used in the present study enhanced mouse embryo development, and the beneficial effects of amino acids at concentrations found in follicular fluid disappeared when essential amino acids were removed from HTF(FF) (data not shown). Lane and Gardner (1997) reported that development of mouse embryos and fetal development after transfer of blastocysts were improved by culture with essential amino acids at the eight-cell stage. Thus, the results presented here demonstrate that supplementation with total amino acids at appropriate concentrations is beneficial to embryo development.

Gardner and Lane (1993) reported that ammonium, an end-product of amino acid metabolism, increases in the culture medium during incubation, and is harmful to embryo development. In the present study, ammonium was produced, but the rate of production was significantly lower in HTF(FF) than in HTF(F-10) and HTF(MEM). Exposure of embryos to high concentrations of ammonium during culture induces fetal retardation and exencephaly (Lane and Gardner, 1994). However, the present results indicate that amino acids at concentrations present in follicular fluid can be safely used for embryo culture. The concentrations of total amino acids and glutamine in Ham's F-10 and MEM are higher than those found in follicular fluid. However, only the concentration of glutamine decreased during incubation in the absence or presence of embryos. Glutamine stimulates somatic cell growth and embryo development (Eagle, 1959; Bae and Foote, 1975; Carney and Bavister, 1987; Chatot *et al.*, 1990; Rieger *et al.*, 1992), but it is unstable in aqueous solution and breaks down spontaneously to ammonium when present at high concentrations (Fürst *et al.*, 1990). Moreover, when amino acids were present at twice the concentration found in follicular fluid, the

improvement of embryo development diminished (data not shown). These results indicate that embryo development *in vitro* is affected not only by the composition of amino acids in the culture media, but also by concentrations of amino acids, particularly glutamine.

In conclusion, amino acids at concentrations present in human follicular fluid promoted blastocyst formation of mouse embryos without high ammonium production. However, as embryo development in mice and humans is affected by other components, such as electrolyte, glucose concentration and osmolarity (Lawitts and Biggers, 1991; Baltz *et al.*, 1991), additional research is needed to establish the optimum medium for human embryo development *in vitro*.

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