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Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters

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The role of amino acids, ethylenediaminetetraacetic acid (EDTA), transferrin, oxygen, glucose, glutamine, taurine and ammonium in CF1 mouse zygote development in culture was examined. Non-essential amino acids and glutamine were shown to alleviate the 2-cell block in culture, and acted in synergy with EDTA to facilitate development to the blastocyst stage. In the presence of amino acids and EDTA, transferrin conferred no beneficial effect. Development of zygotes was significantly impaired if amino acids were removed from the collection medium, even when they were subsequently cultured in the presence of amino acids. Zygote development to the blastocyst stage was significantly improved when modular incubator chambers were used compared to using a conventional incubator, and when an oxygen concentration of 7% was used as opposed to 20%. Addition of taurine to medium containing non-essential amino acids had no effect on embryo development, whereas the removal of glutamine and/or glucose from the culture medium significantly reduced blastocyst cell number. Removal of glucose from the culture medium also resulted in a significant decrease in implantations. Ammonium, generated from the breakdown of amino acids, significantly reduced blastocyst development. EDTA was found to confer its beneficial effects during the first 48 h of culture, and indeed was inhibitory during the second 48 h, resulting in loss of subsequent viability. In summary, the data demonstrate that development of CF1 zygotes to the blastocyst stage is readily achievable. In the presence of non-essential amino acids and glutamine the removal of glucose is detrimental to CF1 mouse embryo development in culture and reduces subsequent viability. Optimal development and maintenance of viability requires more than one culture medium to support the preimplantation period.

Key words: ammonium/culture/EDTA/oxygen/viability

Introduction

Complete development of the mouse zygote to the blastocyst stage in culture has been restricted to a few inbred strains and their F_1 hybrids. Embryos cultured from the majority of mouse strains in conventional embryo culture media such as M16 (Whittingham, 1971) undergo cleavage arrest during the G_2 phase of the second cell cycle, a phenomenon widely referred to as the '2-cell block' (Goddard and Pratt, 1983). Alleviation of the '2-cell block' can be facilitated by several mechanisms, including transfer of embryos to an oviduct (either *in situ* or as an organ culture; Whittingham and Biggers, 1967), transfer of cytoplasm by microinjection from a 2-cell embryo of a non-blocking strain of mice to that of a blocking strain (Muggleton-Harris *et al.*, 1982); inclusion of chelators of heavy metals such as ethylenediaminetetraacetic acid (EDTA) (Abramczuk *et al.*, 1977) and transferrin in the medium (Nasr-Esfahani *et al.*, 1990), which possibly alleviates oxidative stress, increasing the ratio of lactate:pyruvate and the removal of glucose from the medium, at least for the first 72 h of culture (Chatot *et al.*, 1989); low osmolarity, phosphate (0.35 mM) and glucose (0.2 mM) (Erbach *et al.*, 1994); and the omission of phosphate (Scott and Whittingham, 1996). Similarly, in the hamster embryo, the removal of glucose and phosphate facilitated the development of the 2-cell embryo to the 8-cell/morula stage (Schini and Bavister, 1988). However, approaches to overcome cleavage arrest in culture by removing nutrients such as glucose appear rather unphysiological, as glucose is present in mammalian oviduct fluid at significant concentrations (Gardner and Leese, 1990; Nichol *et al.*, 1992; Gardner *et al.*, 1996). Furthermore, a facilitated mechanism for glucose entry into the mouse embryo exists from at least the 2-cell stage onwards (Gardner and Leese, 1988) Although glycolytic activity remains low until around the time of compaction (Wales, 1986; Biggers *et al.*, 1989), glucose is utilized at relatively high rates by the cleavage-stage mouse embryo through the pentose phosphate pathway (O'Fallon and Wright, 1986), presumably to fulfil biosynthetic requirements. Therefore, to alleviate cleavage arrest by removing glucose from the culture medium is not only unphysiological but should be considered as an artefact.

Recent research on the mammalian embryo from several species has determined that those amino acids present at high levels within the oviduct, oocyte and early embryo (alanine, aspartate, glycine, glutamate, glutamine and taurine, Schultz *et al.*, 1981; Miller and Schultz, 1987; Gardner and Leese, 1990) are important regulators of early embryo development in culture This group of amino acids has a striking homology to Eagle's non-essential amino acids and glutamine (Eagle, 1959), with the exception of taurine (Dumoulin *et al.*, 1992a,b), which is not present in tissue culture media. Eagle's non-essential amino acids and glutamine have been shown to decrease the time taken for the mouse embryo to complete the first three cell cycles (Lane and Gardner, 1995a), increase

Table I. Composition (mM) of embryo culture media[a]

Component	mMTF	DM1	DM2	CZB[b]	KSOM[c]
NaCl	103.4	98 4	98 4	81 6	95 0
KCl	4.78	4 78	4 78	4 83	2 50
KH_2PO_4	1.19	1 19	1 19	1 18	0.35
$CaCl_2 \cdot 2H_2O$	1 71	1 71	1 70	1 70	1 71
$MgSO_4 \cdot 7H_2O$	1 19	1.19	1 19	1 18	0 20
$NaHCO_3$	25 0	25.0	25 0	25 1	25.0
Sodium pyruvate	0 37	0.37	0.37	0.27	0.20
Sodium lactate (D/L)	4 79	4.79	4 79	31.3	10.0
Glucose	3.40	3.40	3 40	0.00	0.20
Alanine	0	0 10	0 10	0	0
Asparagine	0	0 10	0 10	0	0
Aspartate	0	0 10	0 10	0	0
Glutamate	0	0 10	0 10	0	0
Glutamine	0	1.00	1 00	1 00	1 00
Glycine	0	0.10	0 10	0	0
Proline	0	0.10	0 10	0	0
Serine	0	0.10	0 10	0	0
EDTA	0	0	0.10	0.11	0.01
Bovine serum albumin	4 mg/ml	4 mg/ml	4 mg/ml	5 mg/ml	1 mg/ml
Phenol Red	0 01g/l	0 01g/l	0.01g/l	0	0

[a]All media were supplemented with penicillin (0.06 g/l) and streptomycin (0.05 g/l).
[b]CZB was supplemented with 5.5 mM glucose after 48 h of culture
[c]KSOM was formulated with L-lactate.

blastocyst formation of zygotes from mouse (Gardner and Lane, 1993a), hamster (Bavister and McKiernan, 1993) and sheep (Gardner *et al.*, 1994) and increase mouse embryo viability (Lane and Gardner, 1994). Furthermore, glycine, when present as the sole amino acid, facilitated mouse embryo development at increased osmotic pressure (Van Winkle *et al.*, 1990). However, it has also been determined that amino acids are metabolized by the embryo and spontaneously break down in the culture medium to produce embryo-toxic amounts of ammonium (Gardner and Lane, 1993a; Lane and Gardner, 1994).

In this study, we examined the effects of non-essential amino acids and glutamine on the in-vitro development of embryos derived from CF1 mice, which exhibit the 2-cell block (Chatot *et al.*, 1989). The effects of other reported regulators of mouse embryo development, such as EDTA, transferrin, oxygen, glucose, taurine and ammonium, were also examined in combination with non-essential amino acids and glutamine. Furthermore, the viability of the resultant blastocysts was determined by transfer to recipient females.

Materials and methods

Media

Zygotes were cultured in medium DM1 (Table I; Lane and Gardner, 1995b). Medium DM1 was supplemented with EDTA (100 μM) (Abramczuk *et al.*, 1977) and bovine transferrin (0.5 g/l) (Nasr-Esfahani *et al*, 1990; Nasr-Esfahani and Johnson, 1992) Embryo collection and handling were performed in HEPES-buffered DM1 (H-DM1) in which 20 mM $NaHCO_3$ was replaced with 20 mM HEPES at pH 7.4.

In each experiment, medium DM1 without any supplements (mMTF, Table I; Gardner and Lane, 1993a) was included as a negative control, i e a medium in which the 2-cell block is manifest. When cultured in medium mMTF, >95% of embryos blocked at the 2-cell stage and none reached the 8-cell stage. In further experiments, the medium CZB (Table I; Chatot *et al.*, 1989) was included as a positive control, i.e a medium which has been shown to alleviate the 2-cell block. For medium CZB, which has no glucose in its formulation, a 1 μl injection of glucose (dissolved in CZB) was added to each 20 μl drop of culture medium after 48 h of culture, to give a final concentration of 5.5 mM (Chatot *et al.*, 1989).

All salts and glucose were of analar grade (BDH, Poole, Dorset, UK). Sodium pyruvate, sodium lactate, glutamine, bovine transferrin, EDTA and Phenol Red were of cell culture grade (Sigma Chemical Company, St. Louis, MO, USA). Antibiotics and amino acid solutions were purchased from CSL (Parkville, Victoria, Australia), Miles Pentex Crystalline BSA (bovine serum albumin, lot 94) from Bayer Diagnostics (Kankakee, IL, USA) and hyaluronidase (bovine testicular) from Sigma.

Embryo collection

Zygotes were collected from 6–8 week old CF1 females Multiple ovulations were induced by an i.p. injection of 5 IU pregnant mare's serum followed 48 h later by 5 IU human chorionic gonadotrophin (HCG). Immediately following the second injection, females were placed with CF1 males of proven fertility. Mating was confirmed by the presence of a vaginal plug the following morning. Cumulus-enclosed zygotes were collected 21 h post-HCG in H-DM1. Zygotes were denuded with 1 mg/ml hyaluronidase in H-DM1 and washed twice in H-DM1 and once in the appropriate culture medium before being placed in culture. To avoid variation between females, embryos from each donor were evenly distributed between treatments.

Culture conditions

Embryos were cultured in groups of 10 in 20 μl of medium under paraffin oil (Labchem, Auburn, NSW, Australia) in 35 mm Primaria Petri dishes (Falcon, Becton Dickinson, NJ, USA). Such conditions provide an optimal embryo/incubation volume ratio for mouse embryo development *in vitro* (Lane and Gardner, 1992). All dishes were gas equilibrated for 6 h prior to culture Embryos were cultured in 5% CO_2 at 37°C for 96 h.

Morphology assessment and cell number determination

Embryo morphology was assessed after 72 and 96 h of culture using phase-contrast microscopy. The following scoring system for development was employed: arrested (embryos at the 2-cell stage), less than morula (uncompacted or partially compacted embryos >2 cells), morula (fully compacted embryos with or without the beginnings of a blastocoel), blastocyst (fully expanded blastocyst with a blastocoel cavity larger than two-thirds of the embryo volume) which may have initiated herniation of the zona pellucida by the trophectoderm. Blastocyst cell numbers were determined after 96 h of culture using a modification of Tarkowski's air drying technique (Tarkowski, 1966). Blastocysts were placed in 0.4% trisodium citrate solution for 5 min and fixed on a precleaned glass slide with ethanol:glacial acetic acid (3:1 v/v). Cell nuclei were stained with Giemsa in Gurr's buffer (pH 6.8) and counted.

Embryo viability

Blastocyst viability was determined by transfer to pseudopregnant recipients, which were 8–12 week old F_1 (C57Bl/6×CBA/Ca) female mice that had been mated with vasectomized males of the same strain. Mating was determined by the presence of a vaginal plug and the day of plug presence was designated day 1 of pseudo-pregnancy. Blastocysts were transferred to the uterine horn of day 4

pseudopregnant recipients CF1 blastocysts flushed from the uterus on day 4 (88 h post HCG) after mating served as in-vivo controls. Into each recipient female, six in-vivo blastocysts were transferred to one uterine horn and six blastocysts from one treatment were transferred to the other horn The in-vivo developed embryos were transferred alternately to the left and right uterine horns On day 15 of pregnancy, implantation, fetal development and fetal weights were assessed

Oxygen analysis

pO_2 was measured using an automated microelectrode analyser (ABL 500 Blood Gas System; Radiometer, Copenhagen, Denmark).

Statistical analysis

For all data collected, the day of transfer was fitted as a factor to remove day-to-day variations. Data for embryo development and viability were assessed using linear-logistic regression where the error distribution was assumed to be binomial. Embryo cell number was determined using log-linear regression where the error distribution was Poisson (Nelder and Wedderburn, 1972). Fetal weights were normally distributed and were examined using analysis of variance Between-treatment differences were determined using the Bonferroni procedure for multiple comparisons (Ludbrook, 1991) Values are expressed as mean ± SEM.

Results

Effect of amino acids in the collection medium

Amino acids have previously been shown to stimulate mouse embryo development in culture (Gardner and Lane, 1993a). An initial experiment was therefore designed to determine whether the presence or absence of non-essential amino acids and glutamine in the collection medium had any effect on subsequent embryo developmental potential. One oviduct from each female was therefore placed into either H-DM1 with or without non-essential amino acids and glutamine Zygotes were then collected and denuded in this medium before culture. Zygotes were consequently exposed to the collection medium for 5 min before culture in medium DM1 supplemented with EDTA (100 μM) and bovine transferrin (0.5 g/l) at 5% CO_2 in air (n = 50 embryos per treatment from a total of three replicates). Significantly ($P < 0.01$) more zygotes collected in the medium containing amino acids developed beyond the 2-cell block to the morula stage (80%) than those collected in the absence of amino acids (58%; Figure 1). Blastocyst formation was also significantly ($P < 0.01$) greater for embryos collected in the presence of amino acids (39%) than in their absence (16%; Figure 1). Furthermore, embryos collected in the presence of amino acids had a significantly ($P < 0.01$) higher blastocyst cell number (31.9 ± 1 4) compared to those collected without amino acids (20.4 ± 1.4). As a consequence of this initial experiment, all embryos were subsequently collected in medium supplemented with non-essential amino acids and glutamine

Effect of individual medium components

After their collection in the presence of amino acids, zygotes were cultured in medium DM1 supplemented with EDTA (100 μM) and transferrin (0 5 g/l) at 5% CO_2 in air. The effect

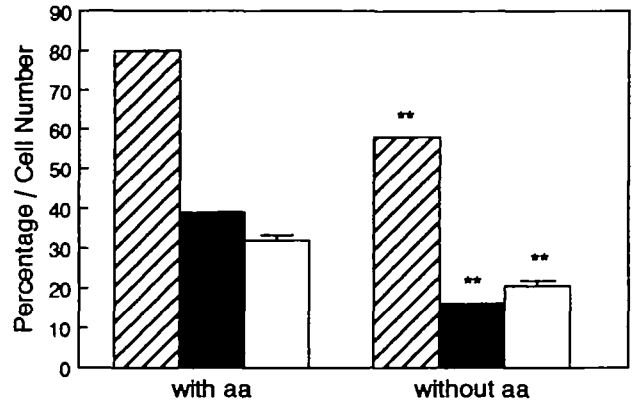

Figure 1. Effect of amino acids in the collecting medium Hatched bars, percentage morula development after 72 h of culture, closed bars, percentage blastocyst development after 96 h of culture; open bars, blastocyst cell number ± SEM, **, significantly different from medium with amino acids ($P < 0.01$).

Table II. Effect of removing individual DM1 medium components on embryo development (n = 110 embryos/treatment from a total of six replicates)

Components present	Arrested at 2-cell (%)	Morula at 72 h (%)	Blastocyst at 96 h (%)	Blastocyst cell number[a]
All supplements[b]	2	74	30	38 1 ± 3.3
No amino acids	46*	2*	0*	–
No EDTA	5	25*	4*	–
No transferrin	3	79	39	37 8 ± 2 4
No bovine serum albumin	3	73	18*	41 2 ± 4 6

[a]Cell numbers are mean ± SEM
[b]All supplements = DM1 plus EDTA (100 μM) and bovine transferrin (0 5 g/l)
*Significantly different ($P < 0$ 01) within a column from medium with all supplements

of removing specific components of the medium was examined to determine their relative effectiveness in supporting embryo development *in vitro* (Table II). Zygotes cultured in DM1 supplemented with EDTA and transferrin were able to develop beyond the 2-cell stage to the morula stage (74%) after 72 h of culture and to the expanded blastocyst stage (30%) after 96 h. Removing amino acids from the medium significantly increased the number of embryos that arrested development at the 2-cell stage. In contrast, removal of transferrin or BSA had no effect on development beyond the 2-cell stage (Table II). Removal of either amino acids or EDTA from the medium resulted in significantly fewer embryos developing to the morula stage (2 and 25% respectively; $P < 0$ 01). Removing amino acids from the medium completely inhibited blastocyst formation, whilst a small percentage developed to the blastocyst stage in the absence of EDTA (4%; $P < 0$ 01). Removal of either bovine transferrin (79%) or BSA (73%) from the medium had no effect on development to the morula compared to medium with all supplements (74%). Blastocyst formation was also not affected by the removal of transferrin (39%), although in the absence of BSA significantly fewer embryos developed to the blastocyst stage (18%) compared to medium with all supplements (30%, $P < 0$ 01). Blastocyst cell number was not

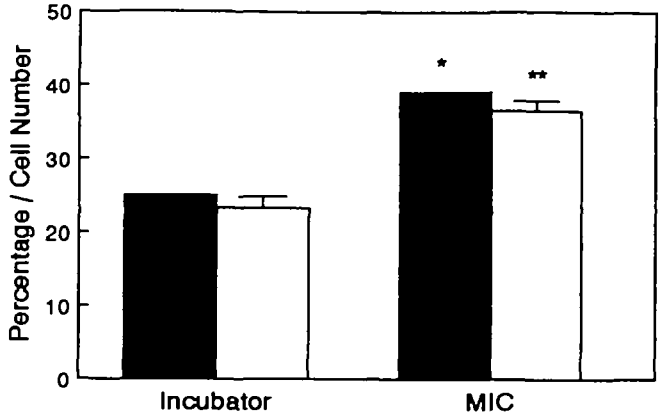

Figure 2. Effect of incubation chamber on embryo development. All embryos were cultured in 20% oxygen. Closed bars, percentage blastocyst development; open bars, blastocyst cell number ± SEM; *, significantly different from incubator ($P < 0.05$); **, significantly different from incubator ($P < 0.01$). MIC = modular incubator chamber.

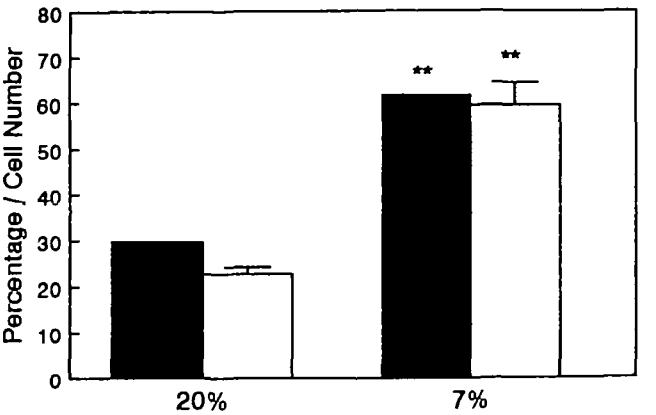

Figure 3. Effect of oxygen concentration on embryo development. Closed bars, percentage blastocyst development; open bars, blastocyst cell number ± SEM; **, significantly different from 20% ($P < 0.01$).

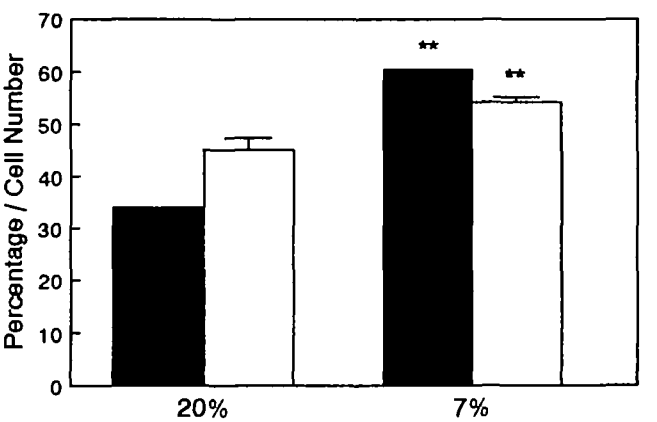

Figure 4. Effect of equilibrating culture media at 20% or 7% oxygen. Closed bars, percentage blastocyst development; open bars, blastocyst cell number ± SEM, **, significantly different from 20% ($P < 0.01$).

affected by the removal of either transferrin or BSA. As a result of this experiment, embryos were subsequently cultured in medium DM1 supplemented with EDTA. This medium was designated 'DM2' (Table I).

Effect of incubation chamber

Zygotes ($n = 140$ from a total of four replicates) were cultured in medium DM2 at 37°C in 5% CO_2 in air for 96 h in either the general laboratory incubator or in a sealed modular incubator chamber (MIC; Billups-Rothenburg, Del Mar, CA, USA), also gassed at 5% CO_2 in air, that was placed inside the incubator. This experiment was designed to investigate the effects of continuously disturbing the gaseous environment of the culture system by the routine opening of the incubator. The ability of zygotes to develop beyond the 2-cell stage was not affected by the incubation chamber. However, significantly more embryos developed to the blastocyst stage when cultured in a sealed incubation chamber compared to an incubator in general laboratory use (opened on average 11 times each day throughout the incubation period; 39.1% compared to 25%; $P < 0.05$) (Figure 2). Blastocyst cell number was also significantly increased in embryos cultured in the sealed chamber (36.6 ± 1.3 compared to 23.2 ± 1.6; $P < 0.01$). Subsequently, all cultures were performed in an MIC.

Effect of oxygen tension

Zygotes ($n = 210$ from a total of five replicates) were cultured in either DM2 or CZB in an MIC placed inside an incubator. The MIC was gassed at either 5% CO_2 in air or 5% CO_2, 7% O_2, 88% N_2 Culturing zygotes in medium DM2 in a reduced oxygen concentration of 7% compared to 20% resulted in significantly more zygotes developing to the blastocyst stage (61.5 and 29.7% respectively; $P < 0.01$). Blastocyst cell number was also significantly increased by culture in a reduced oxygen concentration (59.5 ± 4.9 compared to 22.7 ± 1.6; $P < 0.01$) (Figure 3). Embryos developed in medium CZB also had significantly higher blastocyst development in the presence of a reduced oxygen concentration (45.1 and 29.4% respectively; $P < 0.01$), as well as significantly higher blastocyst cell numbers (41.2 ± 2.5 and 22.6 ± 2.1; $P < 0.01$). Zygotes cultured in medium DM2 gave rise to significantly more blastocysts than those cultured in CZB ($P < 0.05$). Furthermore, the cell number of blastocysts cultured in DM2 was significantly higher than those in CZB ($P < 0.05$). Subsequently, all cultures were performed at an oxygen concentration of 7%.

Effect of equilibrating dishes at high or low oxygen tensions

Culture dishes were equilibrated in an MIC for 6 h at either 5% CO_2 in air or in 5% CO_2, 7% O_2, 88% N_2 prior to culture in 5% CO_2, 7% O_2, 88% N_2. In all, 80 zygotes, from a total of three replicates, were placed in each treatment. Embryos cultured in media that had been equilibrated at a high oxygen tension prior to culture at a low oxygen concentration formed significantly fewer blastocysts than those embryos cultured in media equilibrated at the low oxygen concentration (34 compared to 60.4%; $P < 0.01$) (Figure 4). Blastocysts developed in medium equilibrated at high oxygen also had significantly fewer cells (45.1 ± 2.2 compared to 54.2 ± 1.0; $P < 0.01$). The oxygen concentration of culture medium pre-equilibrated

Table III. Effect of glutamine and glucose on embryo development (n = 40 embryos/treatment from a total of four replicates)

Treatment	Blastocyst (%)	Blastocyst cell number[a]
DM2	50	58 1 ± 1 6
DM2 no glutamine	35	31 8 ± 2 7*
DM2 no glucose	36	42 7 ± 1 9*
DM2 no glutamine or glucose	34	44 3 ± 3 5*

[a]Cell numbers are mean ± SEM
*Significantly different ($P < 0.01$) from DM2

Table IV. Effect (%) of glycine and taurine on blastocyst formation (n = 50 embryos/treatment from a total of five replicates) When embryos were cultured in CZB as a positive control, the rate of blastocyst development was 47 8%

Glycine (mM)	Taurine (mM)			
	0	0 1	1 0	10 0
0 1	71 7 (DM2)	66 0	56 4*	56 5*
1 0	65 1	65 9	60 5	37 2**
10 0	56 8*	49 5*	56 1*	15 6**

*Significantly different ($P < 0.05$) from DM2
** Significantly different ($P < 0.01$) from DM2

at 20% oxygen was measured after incubation for 30, 60, 120 and 240 min in a 7% oxygen environment. By 240 min in a 7% oxygen environment, the oxygen tension in the culture medium was still 15%. Subsequently, for all cultures, medium was equilibrated at 7% oxygen.

Effect of omission of glutamine and/or glucose from the medium

Embryos were collected in H-DM2 without glucose or glutamine. Embryos were then cultured in either (i) DM2, (ii) DM2 without glutamine, (iii) DM2 without glucose, or (iv) DM2 without glutamine and glucose (Table III). Removal of either glutamine or glucose either separately or together did not affect blastocyst formation of the cultured zygotes. However, removing glutamine from the culture medium significantly reduced the blastocyst cell number from 58.1 ± 1.6 to 31.8 ± 2.7 ($P < 0.01$). Similarly, removing glucose from the medium also resulted in a reduction in blastocyst cell number (42.7 ± 1.9; $P < 0.01$). Removing both glutamine and glucose from the medium did not further inhibit blastocyst cell number.

Effect of glycine and taurine

Embryos were collected in H-DM2 and cultured in DM2 with varying concentrations of glycine (0 1–10.0 mM) and taurine (0–10 0 mM) or CZB. Increasing both the glycine and taurine concentrations significantly reduced the percentage of zygotes that developed to the blastocyst stage. There was no interaction between the two amino acids and their effect on blastocyst formation (Table IV) Similarly, increasing concentrations of both glycine ($P < 0.001$) and taurine ($P < 0.001$) significantly decreased blastocyst cell number (Table V).

Table V. Effect of glycine and taurine on blastocyst cell number given as mean ± SEM (n = 50 embryos/treatment from a total of five replicates) When embryos were cultured in CZB as a positive control, the mean cell number was 40 0 ± 3.5

Glycine (mM)	Taurine (mM)			
	0	0 1	1 0	10 0
0 1	57 5 ± 2 1 (DM2)	62 4 ± 2 8*	48 6 ± 2 4*	40.2 ± 2 4**
1 0	55 4 ± 2 6	48 9 ± 2 7*	54 4 ± 2 3	35 6 ± 2 1**
10 0	51 9 ± 2 2*	42 9 ± 1 7**	49 9 ± 2 4*	44 8 ± 2 5*

*Significantly different ($P < 0.05$) from DM2
**Significantly different ($P < 0.01$) from DM2

Table VI. Effect of EDTA and ammonium on embryo development during the second 48 h of culture (n = 60 embryos/treatment from a total of six replicates)

EDTA	Ammonium	Blastocyst (%)	Blastocyst cell number*
+	+	45[ab]	36 0 ± 4 6[ab]
−	+	32[cd]	36 5 ± 1 5[cd]
+	−	68[ac]	42 9 ± 4 0[ac]
−	−	72[bd]	45 8 ± 3 2[bd]

*Cell numbers are mean ± SEM
[a–d]Values with the same superscript letter within a column are significantly different ($P < 0.01$)

Ammonium and EDTA

Zygotes were collected in H-DM2 and cultured for 48 h in DM2, after which time embryos were allocated to one of four treatments (i) medium DM2 which had been preincubated for 48 h at 37°C to facilitate ammonium generation, (ii) medium DM2 which had been preincubated for 48 h at 37°C but lacked EDTA (i.e. DM1), (iii) medium DM2 which had only been pre-equilibrated for 5 h and therefore had low amounts of ammonium, (iv) medium DM2 which had only been pre-equilibrated for 5 h but which lacked EDTA (i.e. DM1). In the presence or absence of ammonium, EDTA had no effect on either blastocyst development or cell number (Table VI) In contrast, the removal of ammonium resulted in a significant increase in both blastocyst formation and cell number irrespective of whether EDTA was present or absent ($P < 0.01$) (Table VI).

Culture of CF1 zygotes in KSOM

Since the inception of this study, a new medium (KSOM; Erbach *et al.*, 1994) has been developed which facilitates the development of zygotes from a CF1×BDF hybrid to the blastocyst in the presence of low phosphate (0.35 mM) and glucose (0.2 mM) (Table I) We therefore examined the ability of this medium to support development of zygotes from a CF1×CF1 mating, compared to medium DM2 in which EDTA was absent for the second 48 h of culture and ammonium toxicity was alleviated by medium renewal at 48 h. Although there was no significant difference in blastocyst development between KSOM and DM2/DM1 (60 versus 71% respectively), embryos cultured in medium DM2/DM1 had a significantly

Table VII. Viability of CF1 blastocysts derived from CF1 zygotes cultured for 96 h (n = 48 blastocysts transferred/treatment from a total of eight replicates; 192 in-vivo blastocysts were transferred)

Treatment	Implantation/ transfer (%)	Fetuses/ transfer (%)	Fetuses/ implantation (%)	Fetal weights (mg)[a]
In vivo[b]	82	81	98	293 6 ± 0.5
DM2/DM2[c]	38[fg]	4	11	161.2 ± 2 1[fh]
DM2/DM1[d]	48[hi]	19*	40*	212.4 ± 0.8[fg]
DM2/DM1-glucose[e]	19[fh]	2 4	12 5	214
CZB	21[gi]	4	20	98.2 ± 1.3[gh]

[a]Fetal weights are mean ± SEM.
[b]In-vivo controls were significantly different to all treatments for all parameters ($P < 0.01$).
[c]DM2/DM2 embryos cultured for 48 h in DM2 and then transferred to fresh DM2.
[d]DM2/DM1· embryos cultured for 48 h in DM2 and then transferred to fresh DM1.
[e]DM2/DM1-glucose. embryos cultured for 48 h in DM2 without glucose and then transferred to fresh DM1 without glucose.
[f,g,h,i]Pairs with the same superscript letter within a column are significantly different ($P < 0$ 05).
*Significantly different to all other culture treatments ($P < 0$ 05)

higher blastocyst cell number (60.0 ± 2.9) compared to medium KSOM (48.6 ± 2.6; $P < 0.05$).

Viability of cultured CF1 blastocysts

Zygotes were cultured in either medium DM2, DM2 without glucose or CZB. After 48 h, half of the embryos cultured in DM2 were transferred to fresh medium DM1 (DM2-EDTA) and the rest were transferred to fresh DM2 medium. Embryos cultured in DM2 without glucose were transferred to fresh DM1 without glucose. After 96 h of culture, blastocysts were transferred to day 4 pseudopregnant recipients. The same percentage of embryos cultured in DM2 and transferred to fresh DM2 were able to implant as those transferred to DM1 (38 compared to 48%); however, significantly fewer of these implantations developed to a fetus (4 compared to 19%; $P < 0.05$) (Table VII). Both the implantation rates and fetal development of blastocysts cultured in CZB were also significantly reduced compared to embryos cultured in medium DM2 for 48 h and then transferred to medium DM1 (Table VII). Furthermore, embryos cultured in the presence of EDTA for the second 48 h (i.e. DM2 or CZB) had significantly reduced fetal weights ($P < 0.05$) compared to those cultured in the absence of EDTA in the second 48 h (DM1, DM1 without glucose) (Table VII). Blastocysts cultured in the absence of glucose resulted in significantly fewer implantations ($P < 0.05$) and decreased fetal development ($P < 0.01$) compared to medium DM2/DM2 or DM2/DM1 (Table VII). Implantation rates, fetal development rates and resultant fetal weights of all cultured blastocysts were significantly reduced compared to in-vivo developed blastocysts.

Discussion

This study showed that Eagle's non-essential amino acids with glutamine and EDTA can independently alleviate the 2-cell block in CF1 mouse embryos in culture. Furthermore, amino acids and EDTA act in synergy to facilitate high rates of blastocyst development. Development through the 2-cell block and formation of blastocysts occurred in the presence of both phosphate and physiological concentrations of glucose (3.4 mM) in the culture medium. Ammonium, generated from the metabolism and breakdown of amino acids, impaired development of CF1 embryos *in vitro*. Interestingly, prolonged exposure to EDTA was not consistent with maintenance of embryo viability after transfer. Rather, the beneficial effects of EDTA were confined to the first 48 h of development. Other factors which had a significant impact on embryo development in culture included the use of an MIC, reduced oxygen environment and the presence of amino acids in the collection medium. Finally, it was demonstrated that CF1 blastocysts developed *in vitro* could give rise to successful pregnancies after transfer to recipient females. Thus this study has highlighted the many interactions which exist between components of a culture system and further emphasized the need to perform embryo transfers in order to ascertain the true effectiveness of modifications to embryo culture media systems.

The medium used for the collection and manipulation of embryos prior to development in culture had a significant effect on the subsequent developmental competence. Omission of amino acids from the collection medium to which the embryos were exposed for <5 min resulted in a significantly lower blastocyst development compared to those embryos collected in the presence of amino acids. It is therefore probable that during this short collection period when amino acids are absent there is a sudden efflux of intracellular amino acids (Schultz *et al.*, 1981), leading to intracellular stress, such as changes in intracellular pH (pHi) or osmotic stress. In support of this hypothesis, Van Winkle and Dickinson (1995) demonstrated that the embryo's intracellular pools of amino acids were affected by the medium used for culture. Amino acids have been implicated as regulators of pHi (Bavister and McKiernan, 1993) and as organic osmolytes (Van Winkle *et al.*, 1990; Lawitts and Biggers, 1992; Biggers *et al.*, 1993), which serve to maintain cellular functions (Somero, 1986). That a period of 5 min of exposure of the zygote to a medium lacking amino acids has such a detrimental effect on subsequent embryo development is of clinical significance, as human IVF oocytes are routinely collected and held in media lacking amino acids.

It has previously been demonstrated that the development in culture of embryos from F_1 hybrid mice is stimulated by culture with Eagle's non-essential amino acids (Eagle, 1959) and glutamine. Specifically, this group of amino acids decreases the duration of the first three cell cycles (Lane and Gardner, 1995a) and increases blastocyst development and cell number (Gardner and Lane, 1993a) and embryo viability (Lane and Gardner, 1994). In our study, the removal of this group of amino acids from culture medium DM1 resulted in a significant proportion of embryos arresting development at the 2-cell stage, with no embryos reaching the blastocyst stage. In contrast, the removal of EDTA from medium DM1 (containing non-essential amino acids and glutamine) did not increase the number of embryos arresting at the 2-cell stage, although its absence resulted in fewer embryos reaching both the morula

and blastocyst stages. Abramczuk *et al.* (1977) were the first to demonstrate that the inclusion of EDTA facilitated the development of zygotes from an outbred strain of mice through the 2-cell block. In their study, however, the medium lacked amino acids, and in it most embryos arrested at the 2-cell stage in the absence of EDTA This difference in medium composition helps to explain the apparent difference between the results presented here. Mehta and Kiessling (1990) subsequently reported that the presence of 100 μM EDTA together with amino acids gave equivalent pregnancy rates to those of in-vivo developed embryos when embryos were cultured from the fertilized oocyte for 44 h before transfer. Of significance in the present study is the synergy between amino acids and EDTA in supporting high rates of development past the 2-cell block and subsequent blastocyst formation, indicating that these medium components are acting through different mechanisms. Similarly, Mehta and Kiessling (1990) found that a combination of EDTA and amino acids was significantly better in maintaining embryo viability than EDTA alone. Presumably, EDTA is functioning predominantly as a chelator, whilst amino acids help support normal cellular functions, though their ability to chelate should not be ignored.

In contrast to the work of Nasr-Esfahani *et al.* (1990) and Nasr-Esfahani and Johnson (1992), the omission of transferrin had no detrimental effect on embryo development. It has been proposed that transferrin chelates free iron in the culture medium, thereby removing it from any possible role in the generation of superoxide radicals. It would therefore appear that EDTA and/or amino acids are a more effective chelating system than transferrin and hence, in their presence, transferrin is not required.

The removal of BSA from culture medium DM1 had no effect on development though the 2-cell block In fact, in the absence of BSA, the same percentage of embryos reached the morula stage at 72 h as those in medium supplemented with BSA. However, only half as many of these morulae produced blastocysts in the absence of BSA. Protein is a major component of oviduct fluid in the mouse (Gardner and Leese, 1990) and has been shown to be taken up by the mouse oocyte (Glass, 1963) and blastocyst (Dunglison and Kaye, 1995). Gardner and Kaye (1991) proposed that BSA may serve as a nutritive source for the embryo. However, Fissore *et al.* (1989) suggested that BSA serves more in a protective role for the embryo.

Changes in medium pH and temperature are known effectors of embryo development. The use of an MIC in this study significantly increased both blastocyst formation and resultant cell number. The beneficial effects of the chambers can be attributed to the maintenance of more constant environmental conditions. This highlights the problems associated with continuous opening of incubators during the culture period.

The observed beneficial effects of culturing embryos at a reduced oxygen tension are consistent with those previously reported for the mouse (Quinn and Harlow, 1978; Umaoka *et al.*, 1992), rabbit (Lindenau and Fischer, 1994), goat (Batt *et al.*, 1991), sheep and cattle (Thompson *et al.*, 1990). Such beneficial effects have been attributed to a decrease in oxidative stress on the embryo. Of interest was the observation that equilibrating culture medium for 6 h at high oxygen tension prior to culture at low oxygen tension was detrimental to embryo development. During the initial period of culture at low oxygen, the embryo will actually be exposed to an oxygen concentration of ~15% oxygen for up to 4 h. This finding confirms the study of Pabon *et al.* (1989), who demonstrated that pre-exposure of mouse embryos to 20% oxygen environment for 1 h prior to culture in 5% oxygen impaired subsequent development. That embryo development is higher in a reduced oxygen tension is consistent with the environment within the female reproductive tract in which the lumenal oxygen concentration is between 1.5 and 8.7% (Mastroianni and Jones, 1965; Mass *et al.*, 1976; Fischer and Bavister, 1993).

The removal of glutamine or glucose from the culture medium suppressed blastocyst formation and significantly reduced cell numbers. However, the removal of both glutamine and glucose had no further negative effect on blastocyst development. These data indicate that the amino acid and carbohydrate may be able to substitute for each other. It has previously been reported that glutamine uptake by mouse embryos is reduced in the presence of glucose (Gardner *et al.*, 1989) and that glutamine is oxidized by the embryo, indicating its use as an energy source (Chatot *et al.*, 1990). The ability of glutamine and glucose to interact as energy sources in somatic cells has been documented (Zielke *et al.*, 1984; Newsholme *et al.*, 1985). More recently, Quinn (1995) has shown that, in the absence of glucose and phosphate, glutamine improves CF1 embryo development. Of significance is the observation that relatively high levels of blastocyst formation can occur in the complete absence of glucose in the culture medium, yet subsequent viability is impaired. Such data highlight the significance of performing embryo transfers following culture. Interestingly, glucose has been reported to have a biphasic effect on the development of the mouse embryo in culture; glucose being detrimental to the development of the mouse zygote during the first 48 h of culture (Chatot *et al.*, 1989; Brown and Whittingham, 1991, 1992) and yet has been shown to be required for subsequent blastocyst formation (Brown and Whittingham, 1991, 1992; Martin and Leese, 1995) Scott and Whittingham (1996) have subsequently shown that glucose is only detrimental to mouse embryos in the presence of phosphate Similarly, Summers *et al.* (1995) showed that glucose is not detrimental to mouse zygote development to the blastocyst in the presence of a low phosphate concentration (0.35 mM). It is clear therefore from the present study that the reported negative effects of glucose on cleavage-stage mouse embryo development are due to its interaction with other medium components, and that even in the presence of high levels of phosphate (1.19 mM) glucose is not inhibitory to the cleavage-stage mouse embryo provided non-essential amino acids and glutamine are present. In support of this observation, Barnett and Bavister (1996) have demonstrated that the inhibitory effects of glucose and phosphate on the development of the 2-cell hamster embryo in culture are significantly diminished in the presence of amino acids, supporting the hypothesis that amino acids are important regulators of embryo development. Regarding the utilization of glucose by the mouse embryo, O'Fallon and Wright (1986) showed that although oxidation was low, utilization of glucose

through the pentose phosphate pathway was at its highest at the 2-cell stage of development. Furthermore, glucose is present in the mouse oviduct at a concentration of 3.4 mM (Gardner and Leese, 1990), and the mouse embryo possesses a facilitated mechanism for glucose transport into the blastomeres from at least the 2-cell stage onwards (Gardner and Leese, 1988). Such data therefore lead one to conclude that glucose does indeed have a physiological function during the cleavage stages of development, even though it cannot be used as the sole energy substrate prior to the fourth cell cycle in the mouse (Biggers *et al.*, 1989). Therefore, the present trend to remove glucose from mammalian embryo culture media (Quinn, 1995) should at best be considered unphysiological. In order to produce viable mouse blastocysts *in vitro*, glucose should be included in the culture medium.

Our laboratory has documented the beneficial effects of Eagle's non-essential amino acids on mouse embryo development (Gardner and Lane, 1993a; Lane and Gardner 1994; 1995a). Dumoulin *et al.* (1992a,b) showed that another amino acid present at high concentrations in oviduct fluid, taurine (Miller and Schultz, 1987), stimulated mouse embryo development to the blastocyst stage. When the effects of this amino acid were determined in the presence of Eagle's non-essential amino acids and glutamine, no further beneficial effect was detected, indicating that taurine's niche was filled by one or more of the other amino acids. Similarly, increasing glycine concentration conferred no beneficial effect, even though Van Winkle *et al.* (1990) showed that when glycine is present as the sole amino acid the optimum concentration was 10 mM. Such data indicate that ability of the mouse embryo to utilize a number of different amino acids to fulfil the same function, plausibly that of osmolytes and regulators of intracellular pH.

Although amino acids have been shown to be key regulators of development *in vitro* and *in vivo* of embryos derived from F_1 mice (Gardner and Lane, 1993a; Lane and Gardner, 1994; Ho *et al.*, 1995), their metabolism and spontaneous breakdown leads to the generation of embryo-toxic amounts of ammonium in the culture medium within 48 h of incubation. Not only does ammonium impair development *in vitro* and *in vivo* but it leads to the induction of the birth defect exencephaly (Lane and Gardner, 1994) Similar to the embryos of F_1 mice, the development of CF1 zygotes *in vitro* was significantly reduced by their exposure to ammonium. The mechanism behind ammonium toxicity has yet to be elucidated. This area of embryo culture is important, as ammonium has been shown to impair the development of embryos of other mammalian species (Gardner, 1994; Gardner *et al.*, 1994).

A new culture medium for the development of CF1×B6D2F1 derived embryos is KSOM (Erbach *et al.*, 1994). When the development of CF1 × CF1 zygotes in this medium was compared to development in DM2/DM1, blastocyst development was equivalent. However, blastocyst cell numbers in embryos cultured in KSOM were significantly lower compared to blastocysts cultured in DM2/DM1. Although there are several differences between the two media with respect to their ionic composition, it is proposed that the inclusion of non-essential amino acids and glutamine could be responsible for the increased development observed in DM2/DM1.

Although EDTA facilitated development of CF1 zygotes to the blastocyst stage, removal of EDTA during the second 48 h of culture did not have an effect on blastocyst development and cell number. It can therefore be concluded that EDTA confers its benefit on the embryo during the first 48 h of culture. In support of this, Hoshi and Toyoda (1985) showed that EDTA conferred its beneficial effects on the embryos of ICR mice before 36 h post insemination. Indeed, Poueymirou *et al.* (1989) observed a delay in the synthesis of transcription-dependent proteins in 2-cell mouse embryos cultured in Whitten's medium when EDTA was absent. We have previously demonstrated that EDTA inhibits glycolysis at the 2-cell stage in CF1 mouse embryos and that this was associated with development through the 2-cell block (Gardner and Lane, 1993b). Similarly, Nakamura *et al.* (1993) showed that exposure of mouse embryos to sodium fluoride, an inhibitor of the glycolytic enzyme enolase, facilitated development past the 2-cell stage. It is therefore feasible that EDTA facilitates mouse embryo development by preventing glucose utilization by the Embden–Meyerhof pathway, thereby reducing the influence of any Crabtree effect (Koobs, 1972), which has been implicated in the induction of embryonic arrest in culture (Seshagiri and Bavister, 1991). In contrast to the cleavage-stage embryo, glycolysis is a major energy-generating pathway at the blastocyst stage and will be required at the time of implantation, when the embryo experiences a period of anoxia prior to the establishment of its own capillary bed within the endometrium (Rogers *et al.*, 1982). Consistent with this hypothesis is the finding that resultant fetal development was significantly impaired when embryos were cultured in the absence of glucose. That the presence of EDTA in the culture medium in the second 48 h also reduced fetal development is supportive of its ability to inhibit glycolysis, the main energy-generating pathway required at implantation. As such, the inhibitory nature of EDTA warrants further investigation.

Fetal development of embryos cultured in medium CZB was significantly lower than in the optimum culture system reported from this study, DM2/DM1. The poor developmental potential of embryos cultured in medium CZB may in part be attributed to the presence of EDTA. Of greater significance is the fact that the conditions which are particularly suited for the cleavage-stage embryo do not necessarily reflect those required by the blastocyst. This is not such a surprising observation when one considers the changes in embryo physiology that occur during the preimplantation period and the different environments that the embryo is exposed to as it progresses along the oviduct to the uterus. Indeed, it has been proposed that the optimal culture system for the development of the zygote to the blastocyst will require not one but several media, each formulated to cater for the changing requirements of the embryo (Gardner and Leese, 1990; Nichol *et al.*, 1992; Gardner and Lane, 1993a; Gardner and Sakkas, 1993; Gardner 1994; Bavister, 1995; Gardner *et al.*, 1996).

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