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Effects of glucose, glutamine, ethylenediaminetetraacetic acid and oxygen tension on the concentration of reactive oxygen species and on development of the mouse preimplantation embryo *in vitro*

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Summary. Analysis over the first 48 h of development *in vitro* from the one-cell stage to the early four-cell stage indicated that (i) ethylenediaminetetraacetic acid (EDTA) exerts the major beneficial effect on culture to the blastocyst stage of F1 and MF1 embryos, (ii) glutamine assists development of MF1, but not F1, embryos to the blastocyst stage and probably functions as part of a metabolic response to oxidative damage to mitochondria and (iii) exposure to glucose at some time during early cleavage is essential for full development to blastocysts. None of the culture conditions examined affected significantly the increase in concentration of reactive oxygen species in late two-cell embryos *in vitro*, although F1 embryos *in vitro* often had lower peroxide concentrations than MF1 embryos. A decline in oxygen tension from 20 to 5% had no consistent effect on culture to the blastocyst stage or production of reactive oxygen species. Aminooxyacetate, an inhibitor of transaminase activity, prevented non-blocking embryos from developing beyond G2 of the second cell cycle. It is concluded that the chelation of transitional metals provides the most effective method of overcoming the block to development *in vitro*.

Keywords: reactive oxygen species; H_2O_2; glucose; glutamine; oxygen; EDTA; mice

Introduction

Fertilized mouse oocytes from most outbred and inbred strains do not develop to blastocysts when cultured in most chemically defined media, but arrest at the two-cell stage, a phenomenon referred to as the two-cell block. In contrast, embryos from certain crosses can develop into apparently normal blastocysts in the same culture medium (Whittingham, 1974; Goddard & Pratt, 1983). Culture conditions (Loutradis *et al.*, 1987) and environment (Quinn & Harlow, 1978) can also influence development. Thus, exposure for the first 48 h to CZB medium (which contains 0·1 mmol EDTA l^{-1}, 1 mmol glutamine l^{-1}, lacks glucose and has a lactate:pyruvate ratio of 116) has been reported to overcome to varying extents the two-cell block in most strains of mouse (Chatot *et al.*, 1989, 1990b). These authors suggested that EDTA alone was ineffective in overcoming the two-cell block in the CF1 strain, that glucose is inhibitory to development over the first 48 h of culture and that glutamine is utilized as an energy substrate in place of glucose via its oxidation to CO_2 through the tricarboxylic acid cycle (Chatot *et al.*, 1990b; see also Carney & Bavister, 1987; Chatot *et al.*, 1989, 1990a; Petters *et al.*, 1990). Thompson *et al.* (1990) showed improved development of sheep

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and cattle embryos through the eight- to 16-cell block in a simple defined medium under lowered oxygen tension. It has also been reported that atmospheric oxygen tension is deleterious to development of one-cell mouse embryos *in vitro* (Pabon *et al.*, 1989), the optimal oxygen requirement being reported as 5% (Quinn & Harlow, 1978). Pabon *et al.* (1989) suggested that oxygen radicals might be the cause of the developmental arrest.

Recently, we reported that the G2–M phase of the second cell cycle *in vitro*, at which the two-cell block occurs, was associated with an increase in reactive oxygen species, such as superoxide and H_2O_2 (Nasr-Esfahani *et al.*, 1990a; Nasr-Esfahani & Johnson, 1991). In the presence of traces of transitional metals such as iron, magnification of peroxidative damage to lipids can occur and H_2O_2 and superoxide can be converted to highly toxic hydroxy radicals (Halliwell, 1987; Halliwell & Gutteridge, 1990). We suggested that these processes might cause developmental arrest, which was overcome by addition of metal chelators to the chemically defined medium (Nasr-Esfahani *et al.*, 1990b; Nasr-Esfahani & Johnson, 1992). In this study, we examined the effect of glucose, glutamine, EDTA and oxygen on the production of reactive oxygen species and on the development *in vitro* of mouse embryos that arrest *in vitro* (MF1) and embryos that do not (F1). We used a measuring technique described in detail previously (Nasr-Esfahani *et al.*, 1990a); 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA), because of its non-ionized state, is membrane permeant and diffuses readily into cells. Within the cell, the acetate groups are hydrolysed by esterase activity forming 2′,7′-dichlorodihydrofluorescein (DCHF), which is polar and thus trapped within the cell. DCHF fluoresces when it is oxidized by H_2O_2 or lipid peroxides to yield 2′,7′-dichlorofluorescein (DCF). The concentration of DCF produced within the cells is related linearly to that of peroxides present.

Materials and Methods

Chemicals

Glutamine was obtained from Flow Laboratories (Rickmansworth, UK); aminooxyacetate and bovine apo-transferrin were obtained from Sigma (UK); EDTA was obtained from BDH (UK). Cylinders of 5% oxygen + 5% CO_2 + 90% nitrogen and 5% CO_2 in air were obtained from BOC (Guildford, UK). Double-distilled water from a Milli-Q system was used in the preparation of media (type GS 0·22 μmol l^{-1}, Millipore, France).

Recovery and handling of oocytes and embryos

MF1 female mice (3–4 weeks old; OLAC, Bicester, UK) and F1 female mice (C57BL/10ScSn/Ola female × CBA/Ca/Ola male, bred in the laboratory) were superovulated by intraperitoneal injection of 5 or 10 iu of pregnant mares' serum gonadotrophin (PMSG; Intervet, Cambridge, UK) and human chorionic gonadotrophin (hCG; Intervet) 48 h apart. Embryos were obtained by pairing females individually overnight with HC-CFLP males (Interfauna, Huntingdon, UK) and inspected for vaginal plugs the next day as an indication of successful mating. Unfertilized oocytes were recovered from unmated females at 24 h after hCG.

Fertilized oocytes at the pronuclear stage were recovered from mated females at 26–30 h after hCG. Embryos were released from the oviduct into warmed H6 + bovine serum albumin (a Hepes-buffered form of modified T6 medium; Nasr-Esfahani *et al.*, 1990b) or H6 + bovine serum albumin (BSA) without glucose and after about 1 h were cultured in drops of T6 + BSA, BAT6 (T6 + bovine apotransferrin at 1 mg ml^{-1}) + BSA or T6 + BSA without glucose (Nasr-Esfahani *et al.*, 1990b) under paraffin oil (FSA Suppliers, Loughborough, UK) ± 1 mmol glutamine l^{-1} and 0·1 mmol EDTA l^{-1} in Falcon tissue culture dishes in 5% CO_2 in air. In some experiments, in which the proportion of oxygen was varied, air-tight modular incubation chambers (Billup-Rothenberg, CA, USA) were used for all oxygen contents tested and were gassed to a slight positive holding pressure to ensure that any equilibration with the atmosphere would be detected. All manipulations were carried out at 37°C on heated stages or pads or in incubators.

Zygotes were cultured under the conditions specified in the results section and inspected at regular intervals as follows: at 47–50 h after hCG, the few one-cell or abnormal oocytes were removed and were not included in the totals, and the remaining embryos scored as two-, three- or four-cells; at 69–72 h after hCG, embryos were scored as >three-cell or compact; at 98–100 h after hCG, embryos were scored as being dead, noncompact, compact, early blastocyst or expanded blastocyst (see Chisholm *et al.*, 1985, for definitions of blastocyst subtype); at 116–119 h after hCG, embryos were scored as being dead, preblastocyst, early blastocyst or expanded blastocyst. In some experiments embryos were transferred from the treatment media to T6 + BSA at 69–70 h after hCG as specified in the Results. In one series of experiments, the passage of embryos from the two-cell stage to the four-cell stage was monitored by hourly observation.

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Measurement of reactive oxygen species

Stock solutions of DCHFDA (Kodak, Eastman Kodak Company, Rochester, NY, USA) were prepared in acetone at 1 mmol l^{-1}. The stock solutions were diluted in H6 + polyvinylpyrrolidone (PVP; BDH Chemicals, Poole, UK) to the required concentration. DCHFDA stock solutions were prepared just before the start of each experiment and were kept in the dark and used for a maximum of 48 h. Embryos were washed thoroughly in H6 + PVP after their removal from T6 + BSA or the condition specified in the Results section and then loaded with the dye for 15 min. The oocytes or embryos were then washed in H6 + BSA to remove traces of the dye and were placed in specially designed small chambers containing H6 + BSA and covered by a coverslip (Maro *et al.*, 1984). The fluorescence emission of the embryos was measured immediately by photocytometry, using a Perspex carrying slide for viewing with a long working distance × 32 objective on a Leitz Ortholux II microscope with stabilized HBO100 mercury-vapour lamp and filter set L1. For quantitation of fluorescence, the photomultiplier housing of a Leitz MPV-1 was fitted to the Ortholux II phototube (McConnell *et al.*, 1990). The phototube contained a variable measuring diaphragm that could be adjusted to surround the periphery of an individual embryo, thus excluding background. A 6·25% transmission neutral density filter (Leitz N16) was placed in the path of the exciting light. Fluorescence emission was deflected to the amplifier/discriminator (Model 1140B, SSR Instruments Co., USA) of a quantum photometer (Model 1140A, SSR Instruments) that had been zeroed against background and set to read in a counts s^{-1} mode via a deflection meter. The individual oocyte or embryo was positioned within the adjusted diaphragm and exposed to the excitation wavelength for <10 s and the fluorescence emission recorded (counts/s^{-1} on the 1 M scale to a maximum reading of 10). For each data point in each experiment, the fluorescence emissions of ten embryos were measured and their mean values were expressed as the mean reading.

This set up, involving brief exposure to the exciting light, was designed to avoid damage to the embryos, which was only detected with exposure for >40 s to the exciting light or removal of the 6·25% transmission neutral density filter. Under these latter conditions, a rapid rise in fluorescence emission, reflecting conversion of DCHF to DCF, was detected as a secondary consequence of light-induced lipid peroxidation. This latter property was used by us as a positive control to show that the uptake and de-esterification of DCHFDA was not limiting the fluorescence signal that we measured under any given condition. Thus, sample embryos were exposed to a brief pulse of high-intensity light and the emission value was recorded (see Nasr-Esfahani *et al.*, 1990a).

Quantification of nuclear DNA in fixed mouse oocytes and embryos

Freshly recovered unfertilized oocytes were freed from their surrounding cumulus complex by exposure to hyaluronidase (0·05%: Type II from ovine testes; Sigma, Poole, UK) in H6 + BSA, followed by three rinses in an excess of H6 + BSA. They were transferred immediately to a fixing solution of 2% formaldehyde in phosphate-buffered saline (PBS made from tablets; Oxoid, Basingstoke, UK) + 0·02% Triton X-100 at room temperature for 30 min and then rinsed and kept in drops of PBS overnight. Blocked two-cell embryos cultured in aminooxyacetate between 24 and 52 h after hCG were similarly fixed. The nuclear chromatin of these fixed cells was stained in 1 ml of freshly prepared DAPI (4′,6 diamidino-2-phenylindole, Sigma; 3·125 µg ml^{-1} in H6) and incubated for 30 min. All the oocytes and two-cell embryos were rinsed three times in PBS and mounted on alcohol-cleaned glass slides and the cellular DNA content was assessed immediately by photocytometry on a Leitz Ortholux II microscope, using filter set A. Readings were taken from the metaphase chromosomes of unfertilized oocytes arrested in metaphase II to obtain a reference value representing 2 C DNA content equivalent to the DNA content of a non-replicated diploid set of chromosomes.

Results

Effect of variation in glucose, glutamine and EDTA on culture and H_2O_2 concentration

Since some experiments involved the omission of glucose from the culture medium, a preliminary experiment was undertaken to determine whether transient exposure to the presence of glucose in the H6 medium used for flushing the one-cell F1 embryos from the oviduct affected their development to the blastocyst stage. The presence of glucose slowed the rate of development of one-cell embryos to the four-cell stage ($P = 0·005$, Table 1), but embryos must be exposed to medium containing glucose at some point within 70 h after hCG (Table 1, groups 2–4) if good rates of blastocyst formation are to be achieved ultimately, even if the exposure to glucose is restricted to the period of recovery from the oviduct (Table 1, group 3). This latter observation confirms that of Brown & Whittingham (1991). In subsequent experiments, glucose was not omitted from the H6 oviductal flushing medium.

MF1 and F1 one-cell zygotes were recovered 28 h after hCG in H6 + BSA and then they were placed in T6, T6 without glucose, ±glutamine (1 mmol l^{-1}) and ±EDTA (0·1 mmol l^{-1}). Embryos

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Table 1. Effect of transient early exposure to glucose on development of F1 mouse embryos to the blastocyst stage

One-cell embryos were recovered at 28 h after hCG in:		Cultured 28–70 h after hCG in:			Cultured 70–118 h after hCG in:	
H6 + BSA − glucose (groups 1 and 2) H6 + BSA + glucose (groups 3 and 4)		CZB (groups 1 and 2) CZB (groups 3 and 4)			CZB (group 1) or T6 (group 2) CZB (group 3) or T6 (group 4)	
Group	Number of embryos	% embryos at 70 h: > three cells	% embryos at 70 h: compact	% Dead or deformed at 96 h	% Blastocysts at 96 h	% Blastocysts at 118 h
1	49	100	100	100	0	0
2	49	100	100	0	88	96
3	44	100	75	5	70	86
4	44	100	71	0	75	98

BSA: bovine serum albumin; hCG: human chorionic gonadotrophin.

were cultured for 48 h, during which some were sampled serially for measurement of H_2O_2 concentrations, the remainder being transferred to T6 medium and then cultured to blastocyst stage (Table 2, Fig. 1). The results showed that (i) the main active agent in promoting successful culture of MF1 and F1 embryos beyond the three-cell stage and through to blastocysts was EDTA (for MF1 embryos, $P = 0{\cdot}0001$ in all comparisons using a two-way contingency analysis; for F1 embryos $P = 0{\cdot}0001$ for 3 versus 7 and 1 versus 5, $P = 0{\cdot}003$ for 11 versus 15 and $P = 0{\cdot}039$ for 9 versus 13); (ii) 1 mmol glutamine l^{-1} was also beneficial for development of MF1 embryos ($P = 0{\cdot}0001$ in all comparisons), but not for F1 embryos ($P > 0{\cdot}2$); (iii) the absence of glucose had no significant effect on the development of one-cell F1 embryos ($P > 0{\cdot}3$), but did improve significantly the development of MF1 zygotes in two of the four comparisons ($P = 0{\cdot}006$ 2 versus 10 and $P = 0{\cdot}0001$ 8 versus 16); and (iv) the rise in H_2O_2 did not differ significantly among groups, although embryos in T6 medium, lacking glucose, but containing glutamine and EDTA, showed consistently a smaller rise in H_2O_2, but this was not significant.

Table 2. Effects of glucose, glutamine and EDTA on the development of MF1 and F1 one-cell mouse zygotes *in vitro*

Group	Strain	Glucose	Glutamine	EDTA	Number of embryos	% > Three cells at 70 h	% Blastocysts at 94 h	% Blastocysts at 120 h
1	F1	−	−	−	93	78	44	72
2	MF1	−	−	−	168	34	0	7
3	F1	−	+	−	108	67	48	67
4	MF1	−	+	−	164	64	1	23
5	F1	−	−	+	109	100	93	97
6	MF1	−	−	+	164	90	16	57
7	F1	−	+	+	110	100	96	99
8	MF1	−	+	+	159	100	53	97
9	F1	+	−	−	41	68	31	73
10	MF1	+	−	−	197	27	0	1
11	F1	+	+	−	41	68	46	78
12	MF1	+	+	−	166	57	2	15
13	F1	+	−	+	41	100	87	93
14	MF1	+	−	+	223	84	10	54
15	F1	+	+	+	44	100	100	100
16	MF1	+	+	+	161	81	37	80

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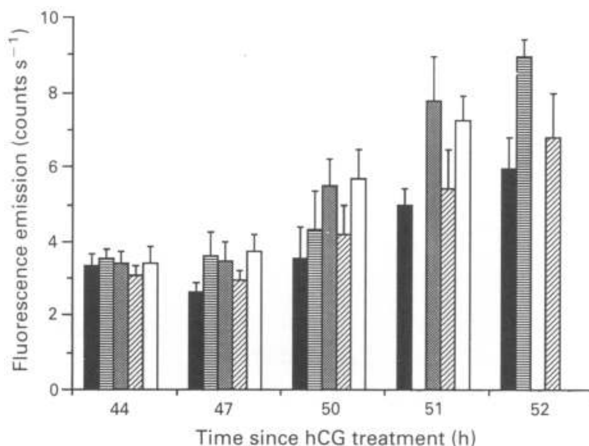

Fig. 1. One-cell fertilized oocytes recovered from MF1 (blocking strain) mice 28 h after injection with human chorionic gonadotrophin (hCG) in glucose-free H6 + bovine serum albumin were cultured in glucose-free T6 medium with glutamine + EDTA (■), glucose-free T6 medium with glutamine alone (▤), or glucose-free T6 medium ▨, T6 medium with EDTA (▨) and T6 medium alone (□) and the fluorescence emission (±SD) proportional to the peroxide concentration in the embryos in each group was measured at the times indicated.

In these experiments, F1 and MF1 embryos *in vitro* benefited from the presence of EDTA, even though F1 embryos are usually regarded as being resistant to arrest *in vitro*. This result was of interest in the light of our previous report that F1 embryos, like MF1 embryos, showed a rise in reactive oxygen species at the late two-cell stage *in vitro* (Nasr-Esfahani *et al.*, 1990a). To determine whether the use of optimal medium prevented the rise in reactive oxygen species in F1 embryos, F1 and MF1 embryos were placed *in vitro* at either the late one-cell (28 h after hCG) or mid two-cell (44 h after hCG) stage and cultured in various media. Embryos recovered at the late one-cell stage were sampled for H_2O_2 measurement at regular intervals and were examined for division to the four-cell stage. Embryos recovered at the mid-two-cell stage were examined only for division to the four-cell stage. MF1 embryos cultured from the late one-cell stage in CZB or BAT6 media did show an improved rate of progress through the second cell cycle and incidence of blastocyst formation, but, compared with the embryos developing *in vivo*, there remained a 2–3 h delay in the transition time through the second cell cycle (Fig. 2b). In contrast, although F1 embryos cultured from the late one-cell stage in T6 alone were retarded, in CZB or BAT6 media their division profile was similar to that of controls developed *in vivo* (Fig. 2a). Direct comparison of reactive oxygen species in F1 and MF1 one-cell embryos, cultured in parallel, revealed in many, but not all, experiments a significantly higher rise in MF1 embryos ($P < 0{\cdot}007$), but the level in F1 embryos did not differ whether optimal or suboptimal medium was used (Fig. 3).

Effect of inhibition of transamination on embryonic culture *in vitro*

Glutamine can serve as the substrate for an alternative pathway for electron transfer to ubiquinone via conversion to succinate, which is then metabolized by succinate dehydrogenase (Fig. 4). To test whether any beneficial effect of glutamine in culture might operate via this route, F1 (non-blocking strain) one-cell embryos were cultured at 30 h after hCG in T6 + BSA at various concentrations of aminooxyacetate, which inhibits transamination of glutamine to 2-oxoglutarate and thereby reduces the amount of succinate. Although low concentrations of aminooxyacetate seemed to stimulate the rate of development to four-cell and blastocysts slightly, at $>0{\cdot}5$ mmol l^{-1} (Table 3), 76–100% of embryos arrested in G2 of the two-cell stage (Fig. 5). When embryos were

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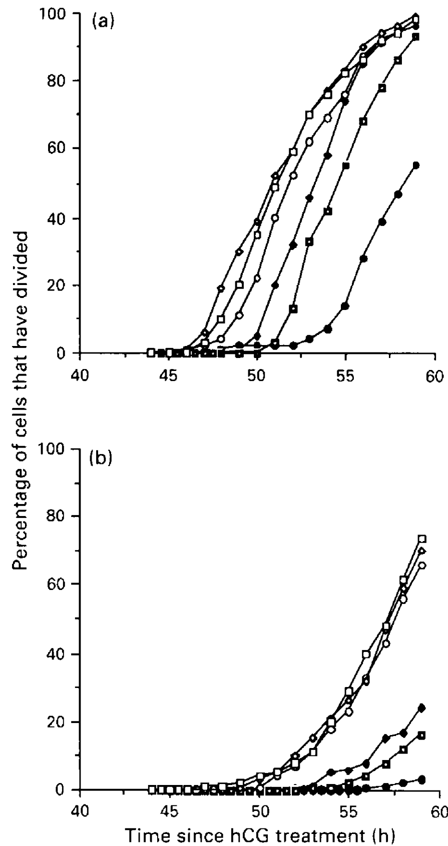

Fig. 2. Cumulative incidence of cell division in one-cell (recovered 28 h after injection with human chorionic gonadotrophin (hCG)) and two-cell (recovered 44 h after hCG) embryos of (a) F1, non-blocking and (b) MF1, blocking mice after culture in T6, BAT6 or CZB medium. Results of representative experiments are shown. (●) T6, late 1-cell; (◆) CZB, late 1-cell; (■) BAT6, late 1-cell. (○) T6, mid 2-cell; (◇) CZB, mid 2-cell; (□) BAT6, mid 2-cell.

cultured in T6 + BSA ± 1 mmol aminooxyacetate l^{-1}, the presence of glutamine at various concentrations did not overcome the aminooxyacetate-induced F1 block (data not shown). The aminooxyacetate block was shown to be specific to the two-cell stage by exposing embryos to 1 mmol aminooxyacetate l^{-1} at various intervals after hCG. Late two-cell and early four-cell embryos recovered at 50 h after hCG and exposed to 1 mmol aminooxyacetate l^{-1} developed normally (Fig. 6a). Aminooxyacetate was only required in the mid G2–M phase of second cell cycle to induce a block in F1 one-cell zygotes cultured *in vitro* (Fig. 6b and c; Bolton *et al.*, 1984).

Exposure to reduced oxygen tension *in vitro* in relation to peroxide production

Exposure of mouse embryos *in vitro* to unphysiological (20%) oxygen concentrations may be deleterious for development, slightly better blastocyst rates occurring at 5% oxygen (Quinn & Harlow, 1978). One-cell fertilized embryos were therefore recovered at 30 h after hCG and immediately placed in 20% or 5% oxygen in airtight modular incubators. Embryos were either cultured through to the blastocyst stage or were assayed for peroxide concentration on the following day. The culture results suggested that 5% oxygen has no clear and consistent beneficial effect on

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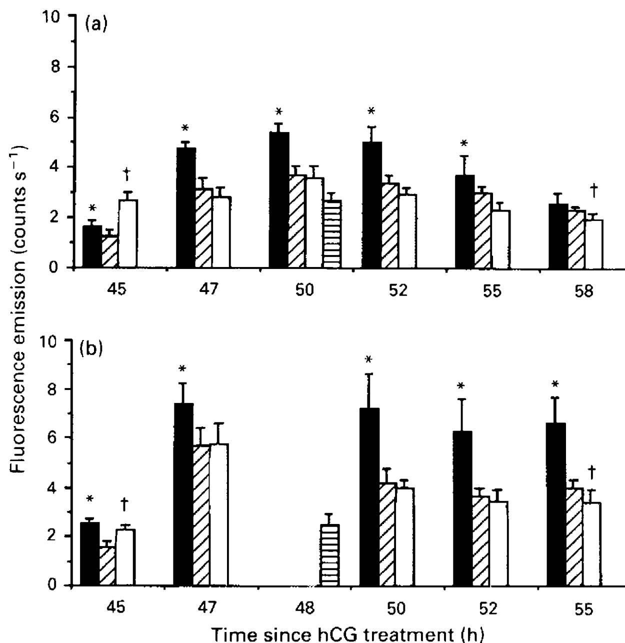

Fig. 3. Mouse embryos in cultures from experiments as illustrated in Fig. 2 were sampled at intervals and the fluorescence emission per embryo (representing reactive oxygen species) was measured. (■) MF1 cultured in T6; (▨) F1 cultured in T6; (□) F1 cultured in CZB; (⊟) freshly recovered F1 embryos. Results from two experiments ((a) and (b)) are shown. *Indicates significant difference between MF1 and F1 embryos cultured in medium T6 (paired *t*-test) and †shows significant difference between F1 embryos cultured in T6 and CZB media ($P < 0{\cdot}007$). Since the latter comparison is only significant at the extremes of the peroxide rise, it may reflect slight differences in the timing of the rise in each population.

incidence of blastocysts (Table 4). When embryos in these experiments were sampled for analysis of H_2O_2 concentrations, the peroxide rise was seen, regardless of oxygen content (Fig. 7).

Discussion

It is important to be able to culture eggs and preimplantation embryos *in vitro* with minimal adverse consequences for long-term development and, if this is not possible, to understand the nature of and the reasons for failure. Although the culture of mouse embryos *in vitro* has been achieved with greater success than for any other species, only recently have media been described that give acceptable results regardless of the strain of mouse providing the oocytes (Chatot *et al.*, 1989, 1990a, b; Nasr-Esfahani *et al.*, 1990b). We have shown that F1 zygotes, hitherto thought to be resistant to arrest *in vitro*, none the less benefit from culture in these media, the rate of progress through the two-cell stage and the incidence of blastocyst formation improving. Likewise, MF1 zygotes, all of which are delayed and most of which arrest *in vitro*, are rescued substantially, but not completely, by culture in these media.

It is clear from our results here and elsewhere (Nasr-Esfahani *et al.*, 1990b) that the principal active property in promoting development in both the beneficial media is the chelating action of EDTA/transferrin, acting externally to the embryos (Fissore *et al.*, 1989; Nasr-Esfahani & Johnson, 1991b) during the mid- to late two-cell stage (Toyoda *et al.*, 1989; Nasr-Esfahani *et al.*, 1990b). We have suggested that chelators might sequester transitional metals such as iron, thereby

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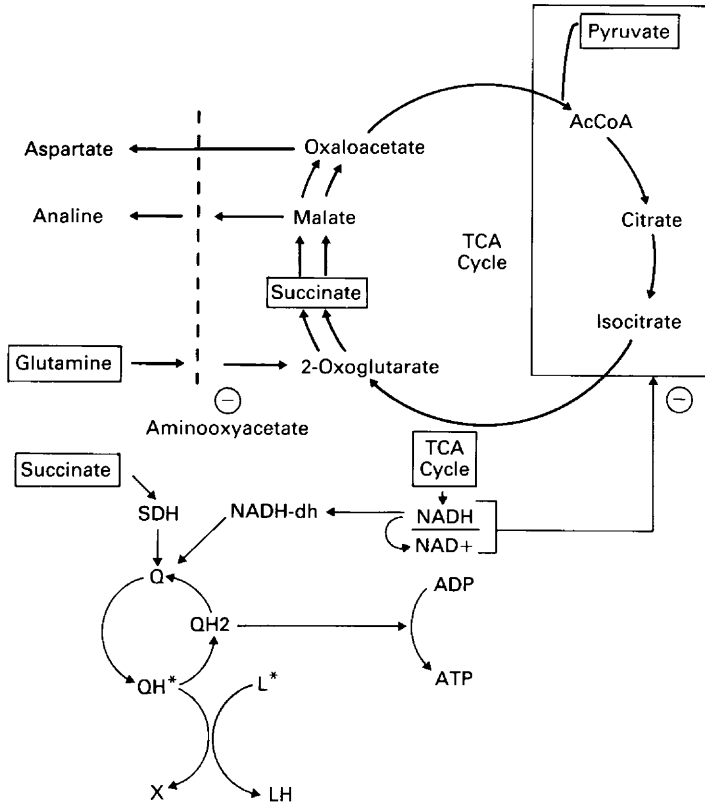

Fig. 4. Schematic representation of the tricarboxylic acid (TCA) cycle and electron transfer via ubiquinone cycle, the input of pyruvate and glutamine, and the effect of aminooxyacetate and lipid peroxidation. The ubiquinone cycle, with ubiquinone (Q), ubisemiquinone (QH*), and ubiquinol (QH2) is shown to be interrupted by lipid radical formation (L*). SDH: succinate dehydrogenase; NADH-dh: NADH dehydrogenase (after Hornsby, 1982).

Table 3. Effect of incubation in aminooxyacetate on the development of F1 one-cell mouse zygotes *in vitro*

Aminooxyacetate concentration ($mmol\ l^{-1}$)	Number of embryos	% Embryos at 70 h		% Blastocysts at 96 h	% Blastocysts at 118 h
		>Three cells	Compact		
0·00	40	65	0	16	65
0·13	34	62	0	13	56
0·25	40	90	0	20	83
0·50	42	93	0	18	78
1·00	42	19	0	12	24
2·00	38	0	0	0	0
4·00	42	0	0	0	0

Embryos were cultured in aminooxyacetate from 28 to 70 h after injection of mice with human chorionic gonadotrophin and they were then transferred to T6 + bovine serum albumin.

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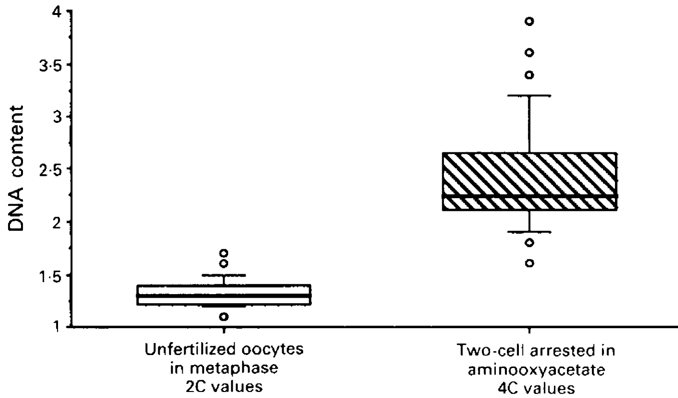

Fig. 5. Box-plots showing the range and distribution of values measured photocytometrically from individual chromosome clusters of 32 mouse oocytes arrested at metaphase II (DNA content = 2C; □) and from 35 two-cell embryos arrested in aminooxyacetate (DNA content = 4C; ▧). The area within each box represents the 95% confidence band and individual points lying outside the 10th (lower) and 90th (upper) percentiles.

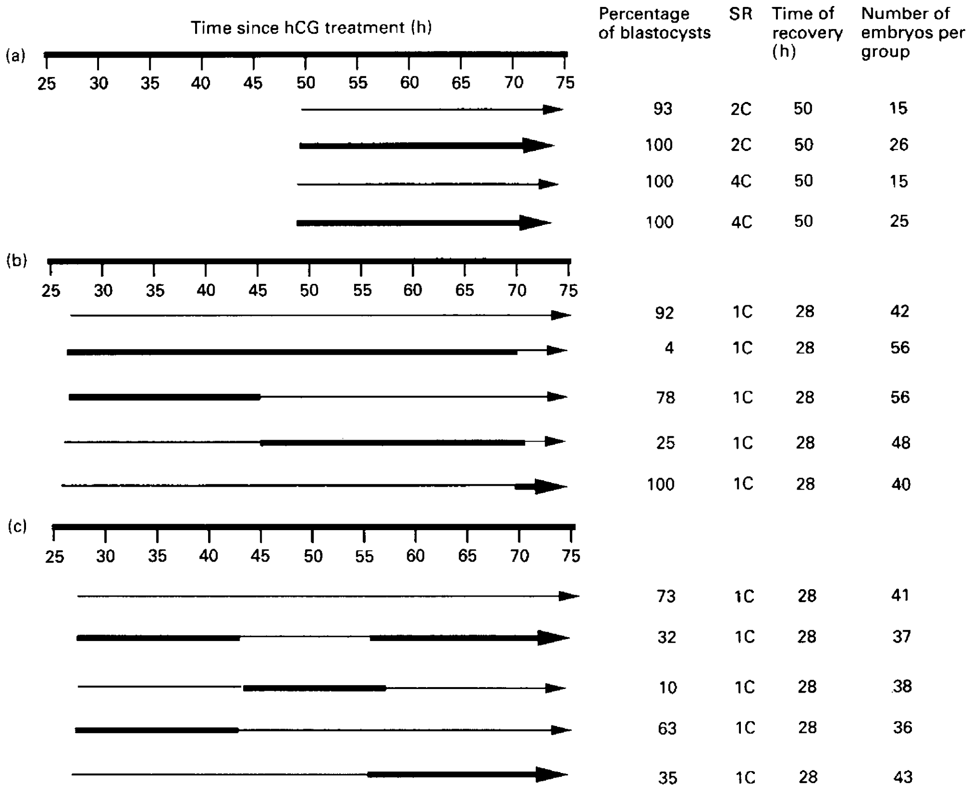

Fig. 6. One-cell (1C), two-cell (2C) or four-cell (4C) F1 (non-blocking) mouse embryos were recovered from the oviduct at 28 or 50 h after injection with human chorionic gonadotrophin (hCG) and placed in culture until 120 h after hCG, when the incidence of blastocysts was recorded. Periods of culture in T6 are indicated by thin bars and in T6 medium containing aminooxyacetate (1 mmol l^{-1}) by thick bars. Results from three experiments (a, b and c) are shown. Note that the G2–M phase of the second cell cycle is delayed by 3–5 h after culture *in vitro* from the one-cell stage in T6 medium (see Fig. 2). SR: stage when recovered.

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Table 4. Effect of oxygen tension on the development of MF1 one-cell mouse zygotes cultured in T6 and BAT6 (at 0·2 mg transferrin ml^{-1}) *in vitro* in modular airtight plastic incubators

% Oxygen in the plastic incubator	Number of embryos	% Embryos at 72 h		% Embryos at 120 h	
		> Three cell	Compact	Compact	Blastocyst
5% Oxygen in T6					
Expt 1	54	41	0	11	4
Expt 2	34	9	0	3	3
Expt 3	44	36	0	0	0
20% Oxygen in T6					
Expt 1	48	19	0	4	0
Expt 2	39	0	0	0	0
Expt 3	22	0	0	0	0
T6-Control*					
Expt 1	50	30	0	0	3
Expt 2	36	31	0	3	3
Expt 3	51	49	0	0	4
5% Oxygen in BAT6					
Expt 1	53	86	0	23	45
Expt 2	42	74	0	2	50
Expt 3	51	90	6	8	91
20% Oxygen in BAT6					
Expt 1	51	78	0	2	40
Expt 2	43	88	0	25	64
Expt 3	42	89	0	19	48
BAT6-Control*					
Expt 1	42	67	0	5	52
Expt 2	39	98	3	3	53
Expt 3	53	90	0	15	48

*Embryos were cultured in continuous-flow incubators in 20% oxygen.

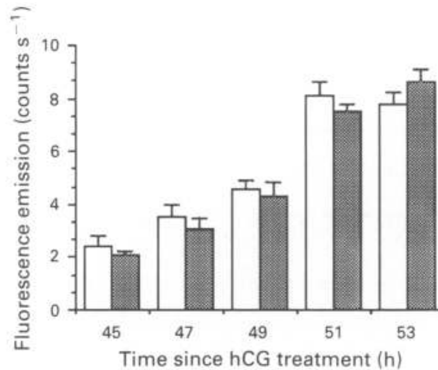

Fig. 7. One-cell fertilized mouse oocytes were recovered 30 h after injection of mice with human chorionic gonadotrophin (hCG) and immediately cultured under 20% (▩, control) or 5% (□) oxygen in airtight modular incubation chambers. On the following day, the embryos from each group were assayed fluorimetrically for peroxides immediately after recovery from these chambers.

limiting iron-catalysed autoperoxidation of lipids and the conversion of superoxide and hydrogen peroxide, concentrations of which are high *in vitro* at the mid- to late two-cell stage, to damaging free radicals (Nasr-Esfahani *et al.*, 1990a, b). However, although the use of chelators in this study improved the development of MF1 embryos *in vitro*, it did not eradicate totally the adverse effects

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of culture. Similar incomplete protection from developmental arrest *in vitro* has been reported after the use of superoxide dismutase (Noda *et al.*, 1991) and reduced glutathione (Legge & Sellens, 1991) to culture media. Moreover, in other studies the efficacy of chelators has varied, depending upon the strain of mouse used or the medium containing the chelator (Fissore *et al.*, 1989). These findings are not surprising since the mode of action of these chelators and scavengers is to limit damage secondary to superoxide production, itself an indicator of the primary defect which must lie in the embryos' metabolic response to culture *in vitro*. Different embryo strains and different culture conditions will presumably influence the nature and magnitude of that response. The suggestion that glucose and glutamine concentrations (Chatot *et al.*, 1990a, b) might influence the effectiveness of culture media offered a possible understanding of the nature of the metabolic response *in vitro*. This possibility was made more attractive by several observations suggesting that a reduction in oxygen tension might also facilitate development, albeit to a somewhat variable extent (Quinn & Harlow, 1978; Legge & Sellens, 1991).

However, the evidence as to whether the presence of glucose is deleterious for early development is ambiguous, indeed the recent, but unexplained, observation that brief exposure to glucose is essential at some point during early cleavage for development to the blastocyst was confirmed (Brown & Whittingham, 1991). The presence of glutamine was beneficial, but only for the more severely affected MF1 embryos. Moreover, the addition of glutamine and removal of glucose did not reduce the concentration of H_2O_2 significantly. Similarly, the inconsistent beneficial effects of low oxygen tension on development were not accompanied by a reduction in reactive oxygen species. Why might these variables be so varied in their effects on culture and how might their limited positive effects be exerted? The variability of response, in this and other studies, to minor variations in culture conditions may simply reflect the fact that no media are yet optimal and that even successful media may achieve their success by a combination of the prevention of primary deleterious responses to *in vitro* culture coupled with adaptive secondary protective responses to any deleterious effects that do occur.

The possibility that the positive effect of a high glutamine:glucose ratio represents an adaptive secondary response is indicated from studies of cells in tissue culture. Thus, an early indication of disturbed mitochondrial function in cells in culture is a shift from oxidative use of pyruvate to use of glutamine (Breen & Shreffler, 1979; Reitzer *et al.*, 1979; Hornsby & Gill, 1981). This transition occurs more rapidly in atmospheric oxygen than in 2% oxygen and is slowed by efficient removal of oxidative species (Hornsby & Gill, 1981; Hornsby, 1982), thereby implicating oxidative damage in the causation of the metabolic transition. Hornsby (1982) suggested that the ubiquinone cycle of mitochondria in cells in culture is interrupted by lipid radicals formed by lipid peroxidation. When ubiquinone activity is impaired, the transfer of electrons from succinate via succinate dehydrogenase occurs preferentially over transfer from NADH via NADH dehydrogenase. The electron flux through succinate dehydrogenase relative to that through NADH dehydrogenase is proportional to flux in the TCA cycle through succinate (the 2-oxoglutarate segment) versus flux through the entire cycle; in turn, this is proportional to utilization of glutamine versus utilization of pyruvate. The $NADH:NAD^+$ ratio controls the relative flux through the two portions of the cycle by inhibiting the pyruvate to 2-oxoglutarate segment. Glutamine conversion to 2-oxoglutarate occurs via glutamine transaminase (Fig. 4). No step between citrate and 2-oxoglutarate is rate limiting in the TCA cycle, but the step between 2-oxoglutarate and malate appears to impede the TCA flux at early embryonic stages (Barbenhenn *et al.*, 1978). Glutamine may therefore boost the flux through this part of the TCA cycle by providing 2-oxoglutarate and thereby succinate. Mouse embryos are known to take up glutamine for use as a metabolic source (Schultz *et al.*, 1981; Gardner *et al.*, 1989; Chatot *et al.*, 1990b), although we do not know the relative proportions of ATP generated from glutamine and from glucose, pyruvate or lactate nor do we know whether it varies under different conditions of culture or with blocking and non-blocking strains. However, it is possible that, when a raised glutamine:glucose ratio is beneficial, it is as a consequence rather than a cause of peroxidative damage and thus functions to aid development in culture downstream

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of the site of protective action of the chelators. The observation that aminooxyacetate, an inhibitor of transamination, can arrest development at the G2 phase of the second cell cycle, an effect limited to that cycle, is consistent with, but does not prove a role for, glutamine in overcoming peroxidative damage. Further supporting evidence for this explanation comes from the observation that embryos have very high intracellular pools of glutamate (Schultz *et al.*, 1981), the precursor of glutamine to which it is converted by glutamine synthetase. This enzyme contains two iron atoms at its active centre and it is highly vulnerable to free-radical-mediated oxidation, thereby decreasing the amount of endogenously produced glutamine available for transamination (Levine *et al.*, 1990; Oliver *et al.*, 1990). Thus, in the light of this evidence, and since glutamine does not reduce the peroxide rise and does not overcome the block in culture completely, whereas iron chelation, alone or with glutamine, can do so (Nasr-Esfahani *et al.*, 1990b and this paper), it is possible that glutamine functions as part of the protective response to mitochondrial damage by residual oxidative species. Mitochondrial abnormalities are known to be present in arrested embryos (Muggleton-Harris & Brown, 1988). In addition to a metabolic protective effect, amino acids such as glutamine can also act effectively as chelating agents (Lindenbaum, 1973; Deighton & Hider, 1989).

If indeed the main active elements in the media that overcome developmental arrest act by limiting oxidative damage, the question remains as to whether prevention of superoxide production could also be effective. Although the reduction in oxygen tension to levels measured *in utero* (Yochim & Mitchell, 1968) provided only limited and variable improvement to culture *in vitro*, it did not prevent the rise in H_2O_2. Since we have recently reported that a primary source of superoxides *in vitro* is likely to be the xanthine–xanthine oxidase system (Nasr-Esfahani & Johnson, 1991), it seems that 5% oxygen tension is adequate for production of superoxide and thus H_2O_2 by this route.

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