The dynamic provision of different energy substrates improves development of one-cell random-bred mouse embryos *in vitro*

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Summary. Preliminary observations showed that one-cell embryos from random-bred MF1 mice avoid cleavage arrest at the two-cell stage ('*in vitro* two-cell block') when cultured in modified M16 culture medium containing lactate and pyruvate but lacking glucose. The roles of lactate, pyruvate and glucose during preimplantation development of embryos from random-bred mice *in vitro* were therefore examined. When all three substrates were present continuously during culture, one-cell embryos arrested at the two- to four-cell stages. Improved development to the morula stage after 96 h in culture was obtained in media containing pyruvate alone, lactate and pyruvate, pyruvate and glucose, lactate pyruvate and glucose for the first 24 h, and medium containing lactate and pyruvate for the remaining 72 h. In a second experiment, embryos were cultured in medium containing pyruvate alone, lactate and pyruvate or pyruvate and glucose for the first 24 h, and lactate plus pyruvate medium for the second 24 h. Subsequent transfer to medium containing lactate, pyruvate and glucose supported the morula to blastocyst transition. These results show that developmental arrest *in vitro* can be overcome by changing the combination of energy substrates at different stages of preimplantation development.

Keywords: preimplantation; embryo; blastocyst; two-cell block; culture; mouse

Introduction

Mouse embryos of all strains can develop into blastocysts when cultured from the late two-cell stage in simple chemically defined physiological salt solutions containing the energy substrates lactate, pyruvate and glucose (Brinster, 1965a, b; Biggers *et al.*, 1967, 1971). However, the embryos of most strains arrest at the two- to four-cell stage when cultured from the one-cell stage, a phenomenon commonly referred to as the '*in-vitro* two-cell block'. Only the embryos of certain inbred strains, and their F₁ hybrids, develop consistently from the one-cell to blastocyst stage under these conditions (Whitten & Biggers, 1968; Biggers, 1971; 1987). Previously, it has been shown that the block may be obviated when arrested embryos are transferred to explanted oviducts in organ culture (Whittingham & Biggers, 1967) or when the cytoplasm from embryos of a non-blocking strain is injected into arrested two-cell stage embryos (Muggleton-Harris *et al.*, 1982; Muggleton-Harris & Brown, 1988).

F₂ hybrid embryos of inbred origin, which do not arrest at the two-cell stage *in vitro*, require glucose for the transition from morula to blastocyst *in vitro* (Brown & Whittingham, 1990b, 1991). Preliminary studies indicated that most one-cell embryos from random-bred MF1 mice avoid

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cleavage arrest at the two-cell stage, and develop into morulae, when cultured in modified M16 mouse embryo culture medium (Whittingham, 1971) containing lactate and pyruvate but lacking glucose (Brown & Whittingham, 1990a). This observation is consistent with the findings of Chatot *et al.* (1989), who developed an improved medium for the culture of one-cell random-bred mouse embryos. The formulation of the medium (CZB) is based on the observation that glucose inhibits development of cultured two-cell (Schini & Bavister, 1988) and eight-cell (Seshagiri & Bavister, 1989a) hamster embryos, and glutamine improves the development of cultured eight-cell hamster embryos (Carney & Bavister, 1987). It is a modification of BMOC-2 (Ebert & Brinster, 1983) with an increased lactate:pyruvate ratio of 116 (Cross & Brinster, 1973), $0.1 \text{ mmol EDTA l}^{-1}$ (Abramczuk *et al.*, 1977), $1 \text{ mmol glutamine l}^{-1}$ (Carney & Bavister, 1987) and no glucose. This medium improves development from the one-cell to morula stage, but the addition of glucose after the first 48 h of culture is necessary for consistent development to the blastocyst stage (Chatot *et al.*, 1989, 1990a, b).

The interactions between substrates are important in meeting the energetic requirements of cultured mouse embryos (Brinster, 1965b). Improved development of one-cell embryos from random-bred mice has been achieved by changing the combination and concentration of energy substrates at various times during culture (Cross & Brinster, 1973). This study examines the specific roles of lactate, pyruvate and glucose (and their interactions), and the possible role of glutamine, at various times during culture of one-cell mouse embryos of random-bred origin.

Materials and Methods

Embryo collection and culture

Fertilized one-cell embryos were obtained from random-bred albino MF1/Lac females mated to MF1/Lac males. The mice were superovulated with intraperitoneal injections of 5 iu pregnant mares' serum gonadotrophin (PMSG; Folligon: Intervet, UK) followed 48 h later by 5 iu human chorionic gonadotrophin (hCG; Chorulon: Intervet, UK). In all experiments, fertilized one-cell embryos were recovered from the oviductal ampullae of mated females between 26 and 27 h after hCG injection. The cumulus cells were dispersed with hyaluronidase (150 iu ml^{-1}) in Hepes-buffered mouse embryo handling medium, M2 (Fulton & Whittingham, 1978; Quinn *et al.*, 1982), and the embryos washed twice in M2 ($2 \times 2 \text{ ml}$). Embryos were cultured in bicarbonate-buffered mouse embryo culture medium, M16 (Whittingham, 1971) or modified M16 in which one or more of the energy substrates (lactate; pyruvate; glucose) was omitted. Media are referred to in the text according to the energy substrates they contain. Culture medium was freshly prepared each week from isotonic stock solutions of the various components so that changes in osmolarity, caused by the omission of energy substrate(s), could be adjusted simply by the addition of extra NaCl stock. The concentrations of lactate, pyruvate and glucose in all media were the same as those in unmodified M16 ($23.28 \text{ mmol sodium lactate l}^{-1}$, $0.33 \text{ mmol sodium pyruvate l}^{-1}$ (Sigma, UK), $5.56 \text{ mmol glucose l}^{-1}$ (BDH, UK)). After washing thoroughly through $2 \times 2 \text{ ml}$ changes of the medium in which they were to be cultured, embryos were placed in small drops ($10 \mu\text{l}$) of the same medium (10 embryos per drop) under light paraffin oil (BDH, UK) in 60 mm plastic tissue culture dishes (Falcon, UK). Medium and oil were previously equilibrated with 5% CO_2 in air at 37°C . Embryos were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C as described previously (Biggers *et al.*, 1971), and developmental progress scored daily. Embryos in all treatments were taken through the washing procedures irrespective of whether they were to be transferred to the same, or different, media for the next culture period.

Experimental design and statistical analysis

Each experiment was repeated three times on three consecutive days, except for Expt 1 which was repeated three times in three separate weeks. Owing to the large number of treatments (36) involved in Expt 1, and thus the considerable amount of washing involved in the transfer of embryos to different media each day, this was divided into three component experiments (12 treatments in each) carried out on different days. We ensured that experimental bias was not introduced by the use of embryos collected from different batches of mice on different days by randomly allocating the treatments in each replicate to the three component experiments using a table of random numbers between 1 and 100 (Fisher & Yates, 1963). A randomized incomplete block design was used, which omitted the glucose-only medium, since preliminary studies showed this could not support first or second cleavage in random-bred MF1 embryos *in vitro* (J. Brown, unpublished observations).

All embryos collected on each day were pooled in the final M2 wash before being allocated to the different treatments. Since no marked differences in development were observed within treatments between replicates, the data

Table 1. The effects of lactate, pyruvate and glucose on development of one-cell MF1 embryos *in vitro*

Treatment number	Medium ^a		Number of embryos ^b	% two-cell after 24 h in culture	Percentage of embryos developing to the various preimplantation stages during 96 h in culture			
	Day 1	Days 2-5			Two-cell	Four-to eight-cell	Morula	Blastocyst
1	L	L	70		69	16	0	0
2	L	P	70		59	3	0	0
3	L	LP	70	53	61	20	0	0
4	L	LG	70		64	11	0	0
5	L	PG	70		76	11	0	0
6	L	LPG	60		67	17	0	0
7	P	L	60		93	27	0	0
8	P	P	60		93	37	0	0
9	P	LP	70	81	83	69	41	0
10	P	LG	70		80	9	0	0
11	P	PG	70		90	11	0	0
12	P	LPG	70		90	59	10	6
13	LP	L	70		93	34	1	0
14	LP	P	70		94	47	1	0
15	LP	LP	60	89	90	75	33	0
16	LP	LG	80		92	32	1	1
17	LP	PG	80		79	20	3	3
18	LP	LPG	60		97	73	22	13
19	LG	L	60		45	7	0	0
20	LG	P	60		60	10	0	0
21	LG	LP	70	55	70	21	4	0
22	LG	LG	60		68	17	0	0
23	LG	PG	70		77	7	0	0
24	LG	LPG	50		86	34	0	0
25	PG	L	80		95	32	10	3
26	PG	P	50		94	60	0	0
27	PG	LP	70	88	93	83	59	9
28	PG	LG	80		89	30	0	0
29	PG	PG	70		93	24	0	0
30	PG	LPG	70		94	77	24	9
31	LPG	L	60		93	23	2	0
32	LPG	P	60		87	32	0	0
33	LPG	LP	70	92	97	81	44	0
34	LPG	LG	50		92	38	4	0
35	LPG	PG	60		95	38	0	0
36	LPG	LPG	70		94	76	17	3

^aEmbryos transferred to medium on day 1, 26–27 h after hCG; day 2, 46–48 h after hCG.

^bResults pooled from three replicate experiments.

L: Lactate; P: pyruvate; G: glucose.

from each were pooled. Differences between treatments were tested by χ^2 analysis of the combined data, using Yates' correction for continuity where d.f. = 1 (Fisher & Yates, 1963).

Results

Experiment 1

In the treatments where pyruvate was available during first cleavage, subsequent development to the morula stage was significantly enhanced by culture in LP medium (treatments 9, 15, 27 and 33) (Table 1). The addition of glucose to each of these treatments after the first 24 h of culture

(except treatment 15) in treatments 12, 30 and 36, respectively, resulted in a significant decrease in the proportion of embryos developing to the morula stage (difference between: treatments 9 and 12; 27 and 30; 33 and 36, $P < 0.001$). The reason that no significant decrease in the level of development was apparent between treatments 15 and 18 ($P > 0.05$) is unclear, although it appears that the presence of lactate during first cleavage may have an adverse effect on later embryonic development (compare treatments 9 and 15, 41% and 33% to morula, respectively).

The presence of glucose, after the first 24 h of culture, therefore, had a marked inhibitory effect on the development of MF1 embryos cultured from the one-cell stage in the presence of pyruvate. This effect was not apparent when glucose was present in combination with pyruvate during the first 24 h of culture (difference in development to morula between treatments 9 and 27, $P > 0.05$). Cleavage to the two-cell stage, after 24 h of culture, was always significantly reduced in the absence of pyruvate ($P < 0.001$). Cleavage to this stage after 24 h in medium with pyruvate alone was significantly lower than that observed in medium with lactate and pyruvate, medium with pyruvate and glucose ($P < 0.01$) or medium with all three substrates ($P < 0.001$).

Experiment 2

Improved development of one-cell MF1 embryos to the morula stage was obtained by culture in medium with pyruvate alone, medium with lactate and pyruvate or medium with pyruvate and glucose for the first 24 h, followed by transfer to medium containing lactate and pyruvate for subsequent culture (Table 1; treatments 9, 15 and 27, respectively). However, development of most embryos was arrested at this stage and only a few (which had been exposed to glucose during the first 24 h of culture; Table 1, treatment 27) developed into blastocysts. A similar 'morula stage block' to development in embryos obtained from B6CBF1 hybrid mice cultured in glucose-deficient medium from the one-cell stage was overcome by the provision of glucose at or before the time of compaction on the third day *post coitum* (Brown & Whittingham, 1990b, 1991). MF1 embryos cultured from the one-cell stage under these three substrate regimens were therefore transferred to complete medium containing glucose after 48 h of culture (72 h post hCG, four- to eight-cell stage) to investigate whether this would support the morula-blastocyst transition. Since many morphologically normal but developmentally retarded morulae were observed at this time, development was also assessed after 120 h in culture (Table 2).

Table 2. Substrate regimens giving maximum development of one-cell MF1 embryos *in vitro*

Treatment number	Medium ^a				Percentage of embryos developing to the various pre-implantation stages during culture					
					96 h				120 h	
	Day 1	Day 2	Days 3-6	Number of embryos ^b	Two-cell	Four- to eight-cell	Morula	Blastocyst	Morula	Blastocyst
1	LPG	LPG	LPG	80	100	83	18	9	20	15
2	LP	LP	LPG	80	96	89	68	23	69	31
3	P	LP	LPG	80	100	80	58	38	58	51
4	PG	LP	LPG	80	99	95	80	39	81	56

^aEmbryos transferred to medium on day 1, 26-27 h after hCG; day 2, 46-48 h after hCG; day 3, 70-72 h after hCG.

^bResults pooled from three replicate experiments.

L: lactate; P: pyruvate; G: glucose.

The proportion of embryos developing to the blastocyst stage in all three experimental treatments, after 96 h in culture, was significantly higher than that observed in control medium with all substrates present (difference between: treatments 1 and 2 ($P < 0.05$); 1 and 3; 1 and 4 ($P < 0.01$)).

The proportion developing to the blastocyst stage in treatments 3 and 4 was not significantly different ($P > 0.05$), demonstrating that the presence of glucose during the first 24 h of culture had no detrimental effect on embryonic development. Indeed, the proportion developing to the morula stage was significantly higher in treatment 4 than in treatment 3 ($P < 0.05$), indicating that the presence of glucose, in combination with pyruvate, during this period has a beneficial effect on development to the morula stage.

Development to the morula stage after 96 h in culture in treatment 3 was not significantly higher than that in treatment 2 ($P > 0.05$), but the proportion developing to this stage in treatment 4 was significantly higher than that in treatment 2 ($P < 0.05$). This shows that, whereas the presence of lactate during first cleavage is not detrimental to embryonic development, a combination of the removal of lactate plus the addition of glucose at this time results in a significant improvement in development.

Many of the morulae observed after 96 h of culture had developed to the blastocyst stage 24 h later. The highest responses were obtained after culture in medium containing pyruvate or pyruvate plus glucose for the first 24 h, medium containing lactate plus pyruvate for the second 24 h, and medium containing all three substrates for the remaining culture period (treatments 3 and 4).

Experiment 3

The effect of glutamine (1 mmol l^{-1}) on development of one-cell MF1 embryos *in vitro* was also examined (Table 3). Medium containing lactate pyruvate and glucose supported development of an unusually high proportion of one-cell MF1 embryos to the blastocyst stage (31%). Typically these embryos arrest at the two- to four-cell stage in this medium (see Tables 1 and 2); but biological variation in experimental material sometimes leads to variation in the response obtained (see Whittingham, 1975). Medium containing lactate and pyruvate supported development of 47% of embryos to the morula stage, but could not substantially support the morula–blastocyst transition (1%). The addition of glutamine to medium containing lactate and pyruvate significantly reduced development to the morula stage ($P < 0.01$). One-cell MF1 embryos failed to cleave in medium containing glutamine as the sole energy substrate, and all degenerated within the first 24 h of culture.

Table 3. Effect of glutamine (1 mmol l^{-1}) on the development of one-cell MF1 embryos *in vitro*

Medium	Number of embryos ^a	Percentage of embryos developing to the various pre-implantation stages during 96 h in culture			
		Two-cell	Four- to eight-cell	Morula	Blastocyst
LPG	130	86	72	45	31
LP	130	92	81	47	1
LP glutamine	130	91	77	28	0
Glutamine	130	0	0	0	0

^aResults pooled from three replicate experiments.

L: lactate; P: pyruvate; G: glucose.

Discussion

Our results showed that exogenous glucose inhibits development of one-cell embryos from random-bred MF1 mice, when present during the second 24 h of culture (two- to four-cell stage),

confirming, in part, the findings of Chatot *et al.* (1989) obtained with embryos from random-bred CF-1 mice. They suggested that the inhibition operated throughout the first 48 h of culture, but did not examine the effect of glucose specifically during the first 24 h. No inhibitory effect was observed during the first 24 h of culture in the present study. Indeed, when present in combination with pyruvate during this period, glucose significantly enhanced development to the morula stage.

In contrast to the findings of Chatot *et al.* (1989) the addition of 1 mmol glutamine l^{-1} to medium containing lactate and pyruvate resulted in a significant inhibition of development. The difference between the two studies may be related to variations in the ability of CF-1 and MF1 embryos to metabolize glutamine. Strain-dependent differences in the ability of mouse embryos to metabolize particular substrates have previously been shown. For example, significantly more B6CBF1 than MF1 embryos undergo first cleavage in medium containing either lactate or glutamine as the sole energy substrate (Expt 3; Brown & Whittingham, 1991). In addition embryos were cultured in an atmosphere of 5% CO_2 in air (Biggers *et al.*, 1971) in the present study, but Chatot *et al.* (1989) used a reduced-oxygen atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 which is thought to favour development from the one-cell stage to blastocyst, at least in culture systems without an oil overlay (Whitten, 1971; Quinn & Harlow, 1978), and manipulation of a single variable can significantly affect the influence of other variables (e.g. energy source and pH; Brinster, 1965a). Additionally, CZB medium contains 31.30 mmol lactate l^{-1} and 0.27 mmol pyruvate l^{-1} , whereas M16 contains 23.28 mmol lactate l^{-1} and 0.33 mmol pyruvate l^{-1} , and changing the type, concentration or combination of substrates significantly affects the influence of other substrates on development (Brinster, 1965b).

The mammalian embryo is exposed to a continually changing environment as it moves along the female reproductive tract, passing from a mainly pyruvate-dominated milieu to a more complex fluid environment after dispersal of the cumulus mass (Donahue & Stern, 1968; Leese & Barton, 1985; Gardner & Leese, 1990). The nutrient requirements also change from a predominantly tricarboxylic acid (TCA) based, to a glycolytic-based metabolism at about the time of compaction and entry into the uterus (Brinster, 1969; Leese & Barton, 1984; Gardner & Leese, 1986). Gardner & Leese (1990) have suggested that optimal development *in vitro* may therefore require two or more media to reflect changing conditions *in vivo*. These results support this suggestion, since greatest development of one-cell random-bred MF1 embryos was achieved with medium containing pyruvate (with or without glucose) for the first 24 h, medium containing lactate and pyruvate for the second 24 h, and medium with all three substrates thereafter.

The concentrations of the substrates were based on the levels found to be optimal for the culture of late two-cell random-bred Swiss mouse embryos by Brinster (1965a, b). They do not necessarily reflect true *in vitro* optima for the different developmental stages, but the beneficial effect of a dynamic substrate regimen is clearly indicated. Further examination of the effect of varying both the combination and concentration of energy substrates at different stages of development will be necessary to produce more appropriate regimens for culturing embryos that arrest *in vitro* during early cleavage. The appropriateness of using high lactate concentrations (20–30 mmol l^{-1}), as in most commonly used embryo culture media, is particularly in question, since both Wales & Whittingham (1973) and Gardner & Leese (1990) have obtained similar developmental responses in the presence of more physiological lactate levels (4.0 mmol l^{-1} and 4.79 mmol l^{-1} , respectively).

The reasons why cleavage to the two-cell stage after 24 h of culture is enhanced by the presence of lactate or glucose, or both, in combination with pyruvate (Expt 1), yet development to the blastocyst stage is reduced if lactate is provided in combination with pyruvate at this stage (Expt 2), are unclear. However, this highlights the caution that must be taken in inferring subsequent viability on the basis of a particular experimental end-point. Caution should also be exercised in inferring that treatments that produce optimum development to the blastocyst stage *in vitro* will also produce optimal viability after implantation.

Lawitts & Biggers (1991) described a radical and innovative method for designing culture media by the technique of simplex optimization (Spendley *et al.*, 1962). They suggest that this will provide

an efficient means of determining the requirements of mammalian embryos *in vitro*, especially those that arrest *in vitro* at various preimplantation stages, and show that optimum development can be obtained in a variety of defined media, the component concentrations of which may bear little relationship to the physiological levels found *in vivo*. However, the postimplantation viability of embryos grown in such media has yet to be established.

The preimplantation embryos of most mammals, including humans, have persistently proved refractory to culture in simple chemically defined media. When designing media, caution must be taken in extrapolating from results obtained in any single species, as emphasized by the recent observation of marked differences in the substrate requirements of mouse and hamster embryos (McKiernan *et al.*, 1991). However, certain common features appear to be emerging. For example, the inhibitory effect of glucose during early cleavage, originally reported in the hamster (Schini & Bavister, 1988), has now been observed in both random-bred mouse (Chatot *et al.*, 1989; Aoki *et al.*, 1990; this paper) and cattle embryos (Ellington *et al.*, 1990), although glucose may be required to support normal development through the later morula–blastocyst transition (Brown & Whittingham, 1991).

Seshagiri and Bavister (1989a, b) have suggested that the inhibitory effect of glucose is due to inadequate energy generation mediated by the 'Crabtree effect' (Crabtree, 1929), whereby glucose stimulates glycolysis, which then competes with mitochondrial respiration for phosphate. However, increasing the phosphate concentration in the medium, which would be expected to overcome this effect (Koobs, 1972), fails to obviate the glucose inhibition to development, a result which they admit is not easily explained by this model. An alternative model is therefore proposed. It is well known that unicellular organisms and cultured cells transcribe specific metabolic genes in response to changes in the availability of different carbon sources. However, many of these genes cannot be expressed if glucose is also present. This phenomenon has been termed 'glucose repression' or 'catabolite repression' (Magasanik, 1961). Recently, it has been shown that transcription of the *fbp-1* gene in *Schizosaccharomyces pombe*, which codes for fructose-1,6-bisphosphatase, is one of the genes subject to glucose repression (Hoffman & Winston, 1991). This may be significant, since fructose-1,6-bisphosphate synthesis is downregulated in cultured mouse embryos prior to the eight-cell stage (Barbehenn *et al.*, 1974). It is therefore possible that 'glucose repression' *in vitro* during the critical period when transcription of the embryonic genome is initiated (Flach *et al.* 1982; Bolton *et al.*, 1984) leads to the inhibition of normal *in vivo* metabolic flux, and that this is why removal of glucose from the medium results in enhanced embryonic development. Glucose repression could also explain the successful rescue of *in vitro* arrested embryos by the injection of cytoplasm from non-blocking embryos (Muggleton-Harris *et al.*, 1982), as the transfer of particular enzymes, their mRNAs, or both, has been proposed as one of the potential rescue mechanisms (Muggleton-Harris & Brown, 1988). Confirmation of this suggestion awaits further analysis at the biochemical and molecular level.

In summary, these results suggest that culture systems should reflect the dynamic changes occurring *in vivo* to achieve the development of viable embryos *in vitro*. Further work is necessary to determine which components, in what concentrations and at which stages, are required to produce viable embryos after culture.

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