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Free radical scavengers ameliorate the 2-cell block in mouse embryo culture

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Mouse zygotes from a strain combination which fails to undergo cleavage *in vitro* (the 2-cell block) were cultured in a variety of media, using either low (5%) or normal (20%) oxygen tension in the gas phase. Development beyond the 4-cell stage was only achieved in medium containing serum and with normal oxygen tension. When serum-free medium was supplemented with free radical scavengers, reduced glutathione (1 mM), but not histidine, catalase or superoxide dismutase, development to the morula or blastocyst stage was promoted in 50% of zygotes cultured with normal oxygen tension. These data suggest that the 2-cell block is a consequence, at least in part, of free radical damage incurred by embryos during collection and culture, and that medium supplementation with the radical scavenger, reduced glutathione, can improve embryo development *in vitro*.

Key words: free radicals/embryo culture/2-cell block

Introduction

The culture of human zygotes is a routine procedure in laboratories supporting in-vitro fertilization and embryo transfer (IVF–ET) programmes. However, the failure rate of this procedure remains stubbornly high and some workers have suggested that damage sustained by the embryo during the culture period is a contributory factor (Braude *et al.*, 1986). Nevertheless, compared with the zygotes of many other species, human embryos appear, at least superficially, to be robust in culture. In the mouse, for example, the zygotes of many genotypes fail to develop *in vitro* beyond the 2-cell stage, a phenomenon commonly referred to as the 2-cell block (Goddard and Pratt, 1983). Since it is likely that the requirements for optimal in-vitro development are similar between different species, culture conditions which overcome the 2-cell block in the mouse might also support optimal development of human zygotes.

Success in overcoming the 2-cell-block in some genotypes was first reported by Whitten (1971), who used a modified Krebs–Ringer bicarbonate medium containing sodium pyruvate and calcium lactate. The novel aspect of this embryo culture system was the pre-equilibration of the medium with a hypoxic gas mixture comprising 5% oxygen, 5% carbon dioxide and 90% nitrogen. Similar hypoxic conditions have subsequently been employed with success in a number of studies (Haidri *et al.*, 1971; Hoppe and Pitts, 1973; Quinn and Harlow, 1978). By contrast, Cross and Brinster (1973) reported the successful culture of random bred Swiss strain zygotes to the blastocyst stage using a normally oxygenated atmosphere of 5% carbon dioxide in air. Although no direct measurements of oxygen tensions have been made in the mouse reproductive tract, values in the region of 8% have been reported in rabbit oviduct (Mastroanni and Jones, 1965) and rat uterus (Yochim and Mitchell, 1968). Thus the hypoxic gas mixture employed by Whitten appears more closely to represent the physiological conditions in which zygotes normally undergo cleavage.

We reasoned that the exposure of embryos, and perhaps of other cultured cells, *in vitro* to 'normal' atmospheric oxygen tensions of 150 mmHg (20%) might be expected to produce oxidative stress with the concomitant generation of free radicals such as singlet oxygen, superoxide anions, hydroxyl radicals and hydrogen peroxide (Fridovich, 1976; Freeman and Crapo, 1982). These free radical species are highly reactive in biological systems where they damage cell membranes, proteins and nucleic acids (Freeman and Crapo, 1982). Because the generation of free radicals is an inevitable consequence of oxidative metabolism, aerobic organisms have evolved scavenging systems to minimize their destructive impact. These comprise, on the one hand, a battery of enzymes (superoxide dismutase, catalase and the glutathione peroxidase/reductase system) and on the other hand, a number of compounds such as tocopherol, β-carotene, reduced glutathione and ascorbate which can quench free radical activity (Freeman and Crapo, 1982; Halliwell and Gutteridge, 1986).

Although the scavenging systems of embryos are clearly equal to their task in the hypoxic developmental environment of the reproductive tract, it seemed to us likely that they might be swamped by excess free radical production in the 20% oxygen atmospheric condition of most embryo culture systems. Thus the 2-cell block might be a result of free radical toxicity associated with the oxidative trauma of zygote collection and culture. In order to test this hypothesis we screened a number of published embryo-culture systems for their efficacy and attempted to alleviate the 2-cell block by incorporating into the most successful system a variety of supplementary free radical scavengers.

Materials and methods

Embryos

In preliminary studies (Legge, 1989) we established that the majority of zygotes produced by BALB/cBy × C57BL/6By

females backcrossed to BALB/cBy males arrested at the 2-cell stage in the standard embryo culture system used in our laboratory (Whitten's medium: Whitten, 1971). Mature (BALB/cBy × C57BL/6By)F_1 female mice (6–8 weeks old) were therefore superovulated by intraperitoneal injections of 12 IU pregnant mares' serum gonadotrophin (PMSG) followed 48 h later by 20 IU human chorionic gonadotrophin (HCG). Following HCG administration, females were placed overnight with BALB/cBy males of proven fertility and checked the following morning for vaginal plugs.

Zygotes, surrounded by cumulus oophorus, were dissected from the oviduct into culture medium prewarmed to 37°C (human tubular fluid, HTF; Quinn, 1985). Oocytes were similarly collected but from females which had not been exposed to males. The cumulus oophorus was removed with bovine testicular hyaluronidase (Sigma, Poole, UK) in HTF at 37°C then zygotes were washed three times in 500 μl of HTF before being transferred to the appropriate culture medium. Fertilization was confirmed by the presence of two polar bodies. Two- and 4-cell embryos were obtained by flushing the oviduct from the ampullary end into a 30 mm culture dish containing HTF. Collection times were as follows: zygotes and oocytes 18 h post HCG, 2-cell embryos 46 h post HCG and 4-cell embryos 56 h post HCG. C57BL/6By embryos, which do not block at the 2-cell stage in our hands, were used as controls.

Culture conditions

The following five established embryo culture media were screened for their ability to support development *in vitro*; Whitten's medium (Whitten, 1971), M16 (Whittingham, 1971), T6 (Quinn, 1982), Earle's medium and HTF (Quinn, 1985). With the exception of Earles medium, which was supplemented with 10% neonatal calf serum (NCS), all media contained bovine serum albumin (BSA; Sigma). Media were prepared using double glass distilled water passed through a Milli-Q water purification system (Millipore, Watford, UK). Freshly prepared media were sterile filtered through 0.22 μm membrane filters (Millipore) and equilibrated by bubbling with one of two gas mixtures: either 5% O_2, 5% CO_2, 90% N_2 or 5% CO_2 in air. For the hypoxic atmosphere, an anaerobe jar (Oxoid, Basingstoke, UK) containing the embryos in culture was equilibrated with the humidified gas mixture for 30 min before sealing. The atmosphere of 5% carbon dioxide in air was maintained in a humidified carbon dioxide incubator (LTE, Oldham, UK). Embryos were cultured at 37°C in 0.5-ml drops of medium under light paraffin oil (BDH, Poole, UK) and were examined for developmental stage at successive 24 h intervals post-HCG up to 96 h.

A total of at least 20 embryos from each stage (zygote, 2-cell, 4-cell) for each medium and gas mixture were cultured in three separate experiments. Separate, freshly prepared batches of medium were used on each occasion. Embryos which showed fragmentation, poor blastomere refractility, or which were otherwise morphologically abnormal were rejected.

Embryo culture in the presence of free radical scavengers

Zygotes were collected into HTF as previously described then cultured in HTF containing one of the following radical scavengers: (i) reduced glutathione (1 mM); (ii) bovine liver superoxide dismutase (SOD, 5000 units/ml); (iii) bovine liver catalase (5400 units/ml); or (iv) histidine (1 mM) (all from Sigma). Development was scored at 96 h post-HCG following culture in 100 μl drops under oil using either the hypoxic (5% O_2, 5% CO_2, 90% N_2) or normally oxygenated (5% CO_2 in air) atmosphere. A minimum of 20 zygotes, divided between three separate experiments, was scored for each culture condition.

Results

Embryo culture

Table I summarizes the development of embryos in Whitten's, M16, T6, Earle's and HTF culture media. In the hypoxic gas mixture none of the embryos progressed beyond the 4-cell stage

Table I. Development of mouse zygotes in five different culture media using two different gas mixtures (5% O_2, 5% CO_2, 90% N_2 or 5% CO_2 in air)

	Development stage[a] (96 h post-HCG)											
Medium	5% O_2					20% O_2						
		1	2	3	4		1	2	3–4	5–8	M	EB
Whitten's	(70)[b]	25	40	4	1	(44)	27	14	3	–	–	–
M16	(40)	4	18	18	–	(40)	14	24	2	–	–	–
T6	(38)	18	12	8	–	(36)	24	10	2	–	–	–
Earle's + NCS	(42)	20	20	2	–	(30)	–	10	4	5	4	7
HTF	(80)	28	44	–	8	(80)	24	36	20	–	–	–
HTF control[c]	(20)	11	9	–	–	(22)	–	–	–	–	8	14

[a]Number of blastomeres, morula (M) or early blastocyst (EB).
[b]Number of zygotes cultured.
[c]Controls are C57BL/6 zygotes (non-blocking).
HTF, human tubular fluid; NCS, neonatal calf serum.

Table II. Development of 2- and 4-cell embryos in different culture media

	Development stage[a] (96 h post-HCG)									
Medium	From 2-cell							From 4-cell		
		2	3	4	8	M	EB		4	M	EB
Whitten's	(20)[b]	6	2	1	2	5	4	(20)	1	7	12
M16	(22)	2	1	4	7	5	3	(20)	1	8	11
Earle's + NCS	(20)	–	–	–	–	–	20	(20)	–	–	20
HTF	(30)	1	2	5	12	6	4	(24)	2	–	22

[a,b]See footnotes to Table I.

Table III. The effects of radical scavengers on the development of mouse zygotes *in vitro*

Medium	Developmental stage[a] (96 h post-HCG)										
	5% O_2				20% O_2						
		1	2	4		1	2	3–4	5–8	M	EB
HTF	(48)[b]	44	4	–	(80)	24	36	20	–	–	–
HTF + SOD	(24)	20	4	–	(26)	3	17	6	–	–	–
HTF + CAT	(30)	23	7	–	(30)	–	21	9	–	–	–
HTF + HIS	(20)	20	–	–	(20)	6	10	4	–	–	–
HTF + GLUT	(42)	38	4	–	(40)	8	–	5	8	5	14

SOD, superoxide dismutase (5000 units/ml); CAT, catalase (5400 units/ml); HIS, histidine (1 mM); GLUT, reduced glutathione (1 mM).
[a,b]See footnotes to Table I.

and the majority underwent at most a single cleavage irrespective of the culture medium used. Embryos of the non-blocking C57BL/6By strain also failed to develop. A similar pattern was observed in 5% CO_2 in air with the majority of embryos in virtually all media failing to develop beyond the 2-cell stage. However, Earle's medium containing 10% NCS supported the development of 67% embryos to the 4-cell stage or beyond with 13% becoming morulae and 27% reaching the early blastocyst stage. Of all the serum-free media, HTF supported the most embryos through at least one round of cleavage (70%). All the 'non-blocking' control embryos (C57BL/6By) reached the morula or blastocyst stage in this medium.

When culture was initiated using later embryonic stages the success rates improved (Table II). Earle's medium supported division for all the 2-cell embryos through to the early blastocyst stage. At least 50% of embryos in each of the other media reached the 8-cell stage but development to the blastocyst was restricted, being <8% in each case. When culture was initiated at the 4-cell stage more than 90% of all embryos proceeded to the morula stage or beyond, with optimal development in Earle's medium in which all embryos became blastocysts.

Culture of zygotes in the presence of radical scavengers

When embryos were cultured in hypoxic conditions (5% O_2) development was severely restricted irrespective of the presence of radical scavengers and no embryos developed beyond the 2-cell stage (Table III). By contrast, when embryos were cultured in 5% CO_2 in air, all media supported some development beyond the 2-cell stage. The presence of histidine did not enhance development but superoxide dismutase (SOD) and catalase promoted an increase in the proportion of zygotes which underwent at least one cleavage. Development was optimal in the presence of reduced glutathione with almost 50% of the embryos reaching the morula or blastocyst stage and 80% of embryos developing beyond the 2-cell stage. Developmental stage was routinely scored at 96 h post-HCG. However, when culture was continued for a further 48 h the majority (> 75%) of blastocysts successfully hatched from the zona pellucida, suggesting that they were developmentally normal.

Discussion

The data summarized in Tables I and II clearly illustrate the phenomenon of the 2-cell block. When media were equilibrated with 5% CO_2 in air, only serum-supplemented Earle's was capable of supporting the development of 'blocking' zygotes beyond the 4-cell stage (Table I). The majority of embyros collected at the 4-cell stage developed to blastocysts in both serum-containing and serum-free media (Table II). HTF, the most successful serum-free medium, by contrast supported development to the morula stage of all zygotes from a closely related 'non-blocking' genotype. Thus developmental arrest at the 2-cell stage cannot be attributed to poor culture conditions.

Interestingly, the use of a 5% O_2-containing gas mixture, which has previously been reported to be optimal for embryo development (Whitten, 1971), was counter-productive. Neither the presence of serum, nor the use of non-blocking zygotes resulted in any development beyond the 4-cell stage. In a comprehensive study of the behaviour of culture media following gassing, we have found that the conditions used in Whitten's study (sealed glass tubes) produce a long-term oxygen tension of 14% in the medium, whereas the use of Petri-dish cultures and an anaerobe jar, as in the current study, maintain an oxygen tension of < 9% (Legge, 1989). This factor, together with detailed differences in medium composition (source and purity of BSA, quality of water) and the medium 'preferences' of different genotypes (Dandekar and Glass, 1987), probably account for the failure of the low oxygen culture conditions in our hands.

Despite our results there is ample evidence from previous studies that hypoxic (though perhaps not so hypoxic as 5–10%) conditions can improve the success of embryo culture (Whitten, 1971; Haidri *et al.*, 1971; Hoppe and Pitts, 1973; Quinn and Harlow, 1978) and that atmospheric oxygen concentrations may be embryotoxic (Pabon *et al.*, 1989). A possible reason for these observations and for the 2-cell block is that the zygote is particularly susceptible to damage by free radicals.

Principal amongst the mechanisms which normally protect against free radical damage is SOD, an ubiquitous enzyme occurring in all aerobic organisms (Fridovich, 1976). Preliminary experiments in our laboratory failed to detect any activity of this enzyme during preimplantation embryo development using the techniques of McCord and Fridovich (1969) and Salin and McCord (1974). However, both oocytes and zygotes showed weak immunoreactivity with a fluorescein iso-thiocyanate (FITC)-labelled anti-human SOD antibody (The Binding Site Ltd, Birmingham) and there was strong binding with the cells of the cumulus oophorus (Legge, 1989). This preliminary finding suggests a role for the cumulus oophorus in protecting the oocyte and zygote from free radical damage.

The product of the dismutation of free oxygen radicals is hydrogen peroxide (H_2O_2), which can cause damage either directly or following its decomposition into highly reactive hydroxyl radicals. In a preliminary study using the fluorescent indicator 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA; Bass *et al.*, 1983), H_2O_2 production could be detected in oocytes and at increased levels in zygotes but not in cumulus oophorus cells (Legge, 1989). Recently Nasr-Esfahani *et al.* (1990) have used a sensitive quantitative version of this technique to show that cultured embryos exhibit a peak of H_2O_2 production at the 2-cell stage during the G_2/M phase of the cell cycle. The timing of this peak of H_2O_2 production is compatible with the idea that free radical damage might contribute to the 2-cell block. The fact that both blocking and non-blocking genotypes show a similar level of H_2O_2 production implies that the defect in 'blocking' genotypes is not the production of excess free radicals, but an impaired system of protective scavengers (Nasr-Esfahani *et al.*, 1990).

Of the radical scavengers tested in the current series of experiments, only reduced glutathione promoted the development of zygotes beyond the 4-cell stage (Table III). Preliminary studies in this department have shown that reduced glutathione supplementation also enhances the apparent growth rate of embryonal carcinoma cells (G.B.Dealtry, personal communication). Reduced glutathione is present at high concentrations in a variety of mammalian tissues where it detoxifies intracellular peroxides (Meister, 1979). It is also involved in decondensation of sperm

nuclear chromatin (Zirkin *et al.*, 1985; Perreault *et al.*, 1988) and in the formation of the male pronucleus (Calvin *et al.*, 1986) so that its presence in the oocyte is well documented. Nevertheless, to our knowledge there have been no investigations of the cytoplasmic concentrations of glutathione in mouse embryos and it is possible, therefore, that relative glutathione deficiency in some genotypes during early cleavage may predispose to free radical damage. The efficacy of exogenous glutathione in ameliorating the 2-cell block suggests that a sufficient quantity gains access to the cytoplasm effectively to scavenge endogenous free radicals.

The 2-cell block does not occur *in vivo* since endogenous production of radicals appears only to be elevated *in vitro* (Nasr-Esfahani *et al.*, 1990). Furthermore, the oviduct is a relatively anaerobic environment (Mastroanni and Jones, 1965; Yochim and Mitchell, 1968; Gosden and Byatt-Smith, 1986) and scavengers (including glutathione and ascorbate) are likely to be present in oviductal fluids. *In vitro*, on the other hand, trace impurities such as copper or iron in chemicals and distilled water and a high concentration of oxygen favour the generation of free radicals. Albumin binds both copper and iron and although the bound metal ions can still participate in Fenton reactions, the hydroxyl radicals produced react at site so that albumin acts as a 'sacrificial antioxidant'. The presence of BSA in culture medium may therefore provide some degree of protection.

The hypothesis that 'blocking' genotypes of mouse zygotes have defective free-radical scavenging systems is consistent with the finding that the injection of cytoplasm from the zygote of a 'non-blocking' F_1 mouse can rescue a known 'blocking' zygote (Muggleton-Harris *et al.*, 1982). Similarly, the microinjection of catalase inhibits endogenous H_2O_2 production in 2-cell embryos (Nasr-Esfahani *et al.*, 1990). Thus supplementation of the free-radical scavenging systems of embryos either by microinjection or, more practically, by medium supplementation, can overcome the 2-cell block. It seems likely that free radical toxicity also contributes to embryonic damage during the culture period necessary for IVF and embryo assessment in human IVF programmes. The routine inclusion of human serum in the culture medium undoubtedly provides a degree of protection, but the data reported here suggest that supplementation with glutathione or other free radical scavengers might be worthy of further consideration.

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