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Hypoxanthine Causes a 2-Cell Block in Random-Bred Mouse Embryos[1]

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ABSTRACT

Ham's F-10, a chemically defined, complex culture medium, commonly used for in vitro fertilization of human as well as animal oocytes, blocked development at the 2-cell stage of > 92% of embryos from random-bred Swiss mice (CD-1), but did not block development of embryos from hybrid-inbred mice (BDF1). In contrast, BWW, a simple, modified Kreb's-Ringer bicarbonate medium, supported development to blastocysts of 85% and 100% of 2-cell embryos from CD1 and BDF1 females, respectively. As little as 15% (v/v) Ham's F-10 added to the BWW blocked the development of the random-bred embryos. Supplementing the BWW with Ham's F-10 components revealed that hypoxanthine (6-30 μM) was responsible for the developmental block to the random-bred embryos. The hypoxanthine block was partially (40%) reversed by adding the chelating agent, ethylenediaminetetraacetic acid. Breeding experiments showed that the hypoxanthine sensitivity of embryos from CD-1 mothers was not affected by the paternal genome.

INTRODUCTION

Early attempts to develop fertilized mouse ova in vitro demonstrated that mouse morulae developed into blastocysts in complex medium (Hammond, 1949) or in simple medium consisting mainly of Kreb's-Ringer bicarbonate (Whitten, 1956; McLaren and Biggers, 1958), but embryos explanted at the 2-cell stage rarely underwent a second cleavage. Subsequent reports that 2-celled embryos developed into blastocysts when the simple medium was supplemented with lactate or pyruvate (Whitten, 1957; Brinster, 1963), allowed the culture of mouse embryos from 2 cells to blastocysts to become a routine procedure in many laboratories. However, development from the 1-celled zygote to the blastocyst stage in vitro remains limited to certain inbred strains of mice and several F-1 hybrids; embryos from random-bred strains explanted at the 1-cell stage undergo the first cleavage to the 2-cell stage, but further development is generally blocked (Whitten and Biggers, 1968; Biggers, 1971; Shire and Whitten, 1980). Breeding experiments (MacLaren, 1981; Goddard and Pratt, 1983) and cytoplasmic transfer experiments (Muggleton-Harris et al., 1982) have shown that the "2-cell block" is due to oocyte cytoplasmic factors and that it is not influenced by the paternal genome.

We have examined embryo development of random-bred (from CD-1 mothers) and hybrid-inbred (BDF2) embryos cultured in Ham's F-10 (Ham, 1963), a complex medium used for in vitro fertilization and early cleavage of human embryos (Edwards et al., 1970, 1980; Trounson et al., 1981; Lopata et al., 1982; Laufer et al., 1984; Quigley, 1985; Veeck, 1985), and in BWW (Biggers et al., 1968), a simple medium in general use for in vitro development of mouse embryos.

MATERIALS AND METHODS

Animals and Breeding

Female mice were 5- to 10-wk-old virgins of two strains: CD-1 (Charles River, Wilmington, MA), a random-bred Swiss strain, and BDF1 (Jackson Labs, ME), an F1 hybrid of two inbred strains, C57B1/6 × DBA/2J. Breeding males (5 to 50 weeks old) were of the same two strains and sources as the females, or were C57B1/6 (Jackson Labs, ME) males of the same age range. Females were superovulated with intraperitoneal injections of 5 IU pregnant mare's serum gonadotropin (Sigma Chemical Co., St. Louis, MO), followed in 48 h with 5 IU human chorionic gonadotropin (hCG; Sigma Chemical Co.), and were housed individually overnight with males. The presence

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of a copulation plug the following morning (17–18 h post-hCG) identified Day 1 of pregnancy. BDF1 females were always bred to BDF1 males; CD-1 females were usually bred to BDF1 males, with the exception of one experiment in which some CD-1 females were bred to CD-1 males and others were bred to C57B1/6 males.

Embryos

Pronuclear-stage zygotes were collected from oviducts 20 h post-hCG into Dulbecco's phosphate-buffered saline (DPBS, Grand Island Biological Co., Grand Island, NY) with 4 mg/ml bovine serum albumin (BSA, Frac. V; Sigma Chemical Co.) and were treated 1–2 min with 67 IU/ml hyaluronidase (Sigma Chemical Co.), rinsed twice, and cultured in groups of 12–23. Embryos with 2 polar bodies and no signs of degeneration were selected for culture. Two-celled embryos were flushed from oviducts 38–40 h post-hCG, rinsed twice, and then transferred to culture conditions.

Embryo Culture

Culture media were prepared fresh biweekly. BWW was formulated as described (Biggers et al., 1968) with freshly distilled Type I (18 megaohm) water and BSA added at 4 mg/ml prior to sterile filtration. Ham's F-10 (Ham, 1963) (powdered, Grand Island Biological Co.) was prepared with freshly distilled, Type I water, and supplemented with 1.35 mM calcium lactate (Calbiochem-Behring Corp., San Diego, CA), 0.095 mg/ml penicillin (Pfizer, New York, NY) and 23 mM Na bicarbonate. Osmolality was adjusted to 280–285 milliosmols.

Components of Ham's F-10 were added to the BWW + BSA in the following groups: 1) heavy metal ions, $FeSO_4$, 3 μM; $CuSO_4$, 10 nM; $ZnSO_4$, 100 nM (Sigma Chemical Co.); 2) amino acids, BME non-essential and essential amino acids (Grand Island Biological) were added in concentrations equivalent to Ham's F-10 formulation, with the exception of cystine (absent from Ham's F-10), glutamine (1–10 times the concentration in Ham's F-10), and isoleucine, leucine, lysine, methionine, and tyrosine, which were at 0.2–2 times the concentration in Ham's F-10; 3) vitamins, BME vitamins were added at 0.3–3 times the concentrations in Ham's, with the exception of biotin, which was 40 times the concentration; 4) lipoic acid (Sigma Chemical Co.) and 5) thymidine (Sigma Chemical Co.) were added at the concentration in Ham's F-10; 6) hypoxanthine (Sigma Chemical Co.) was added at 30 μM, the concentration in Ham's F-10, or at the concentration in 10%, 15%, or 50% (v/v) Ham's F-10 in BWW + BSA, as indicated. "Mock" Ham's F-10 was BWW + BSA, plus the first five groups of components described above.

Human fetal cord blood, collected at Caesarean section, was centrifuged immediately, and the plasma was removed and allowed to clot. The serum recovered from the clot was sterile-filtered (Falcon, 0.2 μm nitrocellulose filter), and heated to 56°C for 30 min. The Ham's F-10 was used for embryo culture either without any protein supplementation or with 10% serum. Culture media were pre-equilibrated overnight at 5% CO_2 in air, 37°C, $\geq$ 95% humidity with pH = 7.35–7.44.

Statistics

The results were statistically analyzed by Student's *t*-test and Fisher's Least Significant Difference Test.

RESULTS

Approximately 90% of zygotes from the F-1 hybrid females developed to the morula stage in vitro in either the simple or the complex media (Table 1). Development of these F-2 hybrid embryos to 2-cell and morula stages was not significantly different ($p>0.05$) for the 3 culture media, and there were no significant differences in development to the blastocyst stage between BWW + BSA and Ham's F-10 ($p>0.05$), but the addition of 10% human cord serum significantly decreased blastocyst development ($p<0.05$). In contrast, CD-1 embryos cleaved to the 2-cell stage equally well in BWW + BSA and Ham's F-10 ($p>0.05$), but the first cleavage was depressed by the addition of 10% cord serum ($p<0.06$). In addition, although 60% of zygotes from the random-bred mothers developed to the morula stage in the BWW + BSA, fewer than 5% developed beyond the 2-cell stage in the Ham's F-10 (Table 1). The addition of 10% serum to the Ham's F-10 did not improve the development of the random-bred embryos, and actually inhibited development of the hybrid-inbred embryos from the morula to the blastocyst stage.

Embryos explanted at the 2-cell stage were also monitored for development in the simple versus the complex media. All of the 2-celled F-1 hybrid embryos developed into blastocysts in the BWW + BSA, a result that was not affected by the addition of

TABLE 1. Development of pronuclear stage mouse embryos in simple and complex culture media.[a]

Culture medium	BDF1[c] 2-C	BDF1[c] M	BDF1[c] B	CD-1[c] 2-C	CD-1[c] M	CD-1[c] B
BWW + BSA	82 (± 11)	94 (± 4)	63 (± 14)	93 (± 2)	62 (± 6)	39 (± 2)
Ham's F-10	86 (± 2)	93 (± 4)	72 (± 4)	77 (± 12)	5 (± 3)	0 (± 0)
Ham's F-10 + serum	91 (± 3)	91 (± 3)	33 (± 2)	64 (± 11)	12 (± 6)	2 (± 2)

[a]Embryos (190) were scored for development after 24 h of culture (2-cell stage) and 96 h of culture (Day 5 of pregnancy, blastocyst stage in vivo).

[b]The percentages shown are the means of three to six replicate conditions (12–18 embryos each) repeated in three separate experiments of 4–6 females each. Numbers in parentheses are standard errors of the mean. Percentage to morula (M, ≥8-cells with no degeneration) and blastocyst (B, trophoblast-enclosed blastocoel, hatched or nonhatched from zona pellucida) stages are percentage of 2-cell (2-C) stage embryos.

[c]Strain of mother.

15% (v/v) Ham's F-10 (Table 2); 79% of the F-1 hybrid embryos developed into blastocysts in the Ham's F-10. A high percentage (85%) of the 2-celled random-bred embryos also developed into blastocysts in BWW + BSA, but ⩽ 8% developed beyond the 2-cell stage in the Ham's F-10, with or without BSA, or in BWW + BSA with as little as 15% (v/v) Ham's F-10 (Table 2).

To determine if the Ham's F-10 was toxic to embryos from CD-1 females at all stages of development, 2-celled embryos were cultured for 24 h in the BWW + BSA, and subsequently transferred at the 4- to 8-cell stage into Ham's F-10. Seventy percent of the control embryos that were not transferred, and 50% of the embryos transferred to the Ham's F-10, continued development to blastocysts.

CD-1 females were superovulated by routine procedures, and mated with either CD-1, hybrid-inbred (BDF-1), or inbred (C57B1/6) males to determine if the paternal component of the 2-celled embryos influenced the developmental block in Ham's F-10. The results (Table 3) were that the paternal component of the embryos from CD-1 females did not influence the block at the 2-cell stage in Ham's F-10.

To determine which components in the complex media were responsible for the developmental block, the components of Ham's F-10 were divided into 6 groups: 1) metal ions, 2) essential and nonessential amino acids, 3) vitamins, 4) lipoic acid, 5) thymidine, and 6) hypoxanthine. Supplementing the BWW + BSA with the first 5 groups of components of Ham's F-10, individually or collectively ("Mock" Ham's F-10), did not significantly affect blastocyst development of 2-celled embryos from CD-1 females (Table 4).

In sharp contrast was the effect of hypoxanthine: supplementing the BWW + BSA with hypoxanthine resulted in a dose-dependent developmental block of the random-bred embryos (Table 4); 30 μM and 15

TABLE 2. Development of 2-celled mouse embryos in simple and complex culture media.[a]

Culture medium	Percent development[b] BDF1[c] M	BDF1[c] B	CD-1[c] M	CD-1[c] B
BWW + BSA	100 (± 0)	100 (± 0)	87 (± 3)	85 (± 3)
Ham's F-10	86 (± 0.3)	79 (± 4)	9 (± 3)	8 (± 3)
Ham's F-10 + BSA	–	–	6 (± 2)	4 (± 2)
BWW + BSA + 15% Ham's F-10	100	100	7 (±¾)	6 (± 4)

[a]Embryos (507) were scored for development to morulae (M) or blastocysts (B) after 72–74 h of culture.

[b]Percentages are the means of two to three replicate conditions (12–20 embryos each) repeated in 3–14 separate experiments of 3–4 females each, with the exception of the BWW/BSA/Ham's F-10 condition with BDF2 embryos, which was a single experiment.

[c]Strain of mother.

TABLE 3. Development to blastocysts of 2-celled hybrid embryos of CD-1 females mated to random-bred (CD-1), F-1 hybrid (BDF1), and inbred (C57B1/6) males.[a]

Culture medium	Percent development[b]		
	CD1[c]	BDF1[c]	C57B1/6[c]
BWW + BSA	81 (± 19)	86 (± 5)	68 (± 7)
Ham's F-10	6 (± 6)	9 (± 3)	6 (± 2)
Ham's F-10 + BSA	5 (± 5)	5 (± 2)	4 (± 4)

[a]Embryos (293) were scored for development to blastocysts after 72–74 h of culture.

[b]The percentages are means of two to three replicates (CD-1 and C57B1/6 matings) or four to ten replicates (BDF1 matings) of embryo cultures containing 14–17 embryos each.

[c]Strain of father.

μM hypoxanthine, equivalent to the concentrations in 100% and 50% Ham's F-10 in BWW + BSA, respectively, blocked 92% of 2-celled embryos from CD-1 mothers; 6 μM, equivalent to 20% Ham's F-10 in BWW + BSA, blocked 83% of the embryos at the 2-cell stage. Supplementing the "mock" Ham's F-10 with 30 μM hypoxanthine blocked 65% of the 2-celled embryos, a result significantly different from the 89% blastocyst development in the "mock" Ham's F-10 with no hypoxanthine. However, the 35% development to blastocysts in the "mock" Ham's F-10 plus hypoxanthine was also significantly different from the 9% development in the Ham's F-10, suggesting that a component in the "mock" Ham's F-10 may have partially neutralized the hypoxanthine effect, or that other, unknown inhibitory substance(s) were present in the preformulated Ham's F-10. Delaying embryo collection to the late 2-cell stage (44–46 h post-hCG) resulted in 55 (± 9)% development to blastocysts in the presence of hypoxanthine, indicating that the hypoxanthine was less inhibitory to late 2-cell random-bred embryos. The development of the BDF2 hybrid-inbred embryos was not inhibited by 30 μM hypoxanthine.

To determine if the developmental block to the random-bred embryos could be due to trace metal contamination of the hypoxanthine, or the Ham's F-10, embryos were cultured in the presence of ethylenediaminetetraacetic acid (EDTA). The percentage of embryos progressing to the blastocyst stage was, at most, 40–49% at all EDTA concentrations tested in the Ham's F-10 and in the BWW + BSA with 30 μM hypoxanthine (Fig. 1). Although this development was significantly greater than the development without the EDTA ($p<0.005$), it was significantly less than the 92% development to blastocysts observed in the BWW + BSA with EDTA and no hypoxanthine.

TABLE 4. Development of 2-celled embryos cultured from the 2-cell stage in complex medium and in simple medium supplemented with components from complex medium.[a]

Culture medium	Percent development[b]	
	CD-1[c]	BDF1[c]
Ham's F-10	9 (± 3)*	79 (± 4)
BWW + BSA	83 (± 5)***	100 (± 0)
+ 10% Ham's F-10	35 (± 12)**	ND
+ 15% Ham's F-10	6 (± 4)*	ND
+ Fe^{++}, Cu^{++}, Zn^{++}	90 (± 5)***	ND
+ Amino acids	76 (± 8)***	ND
+ Amino acids, vitamins	83 (± 7)***	ND
+ Lipoic acid	86 (± 14)***	ND
+ Thymidine	86 (± 10)***	90 (± 1)
+ Hypoxanthine, 30 μM	8 (± 2)*	91 (± 1)
+ Hypoxanthine, 15 μM	8 (± 1)*	ND
+ Hypoxanthine, 6 μM	17 (± 7)*,**	ND
+ "Mock" Ham's F-10 − hypoxanthine	89 (± 3)***	88 (± 12)
+ "Mock" Ham's F-10 + hypoxanthine	35 (± 5)**	ND

[a]Embryos from CD-1 (872) and BDF (195) females were scored for development to blastocysts after 72–74 h of culture.

[b]Percentages are means of three to fifteen replicates of embryo cultures containing 14–17 embryos each. Values with the same superscript are not significantly different, and are significantly different ($p<0.05$) from values with different superscripts by the Fisher's Least Significant Difference Test.

[c]Strain of mother.

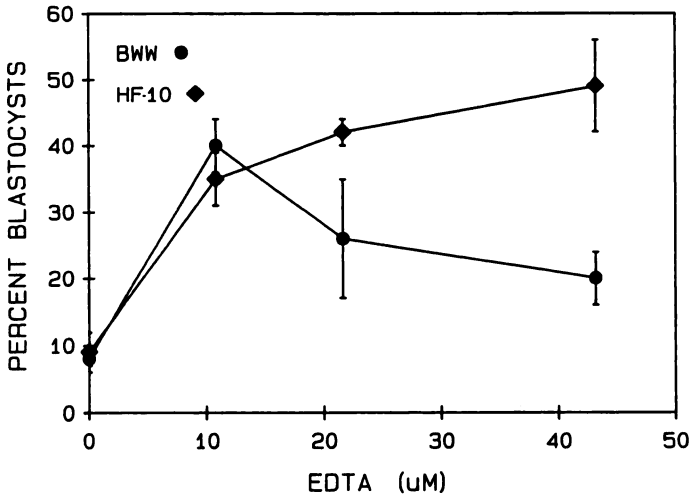

FIG. 1. The effect of EDTA on the development of random-bred embryos cultured in 30 μM hypoxanthine. Embryos (95) were recovered from pregnant CD-1 females. The data are means of two to four replicates of embryo cultures containing 12–20 embryos each. *BWW* = simple medium; HF-10 = Ham's F-10, complex medium.

DISCUSSION

The results presented here show that 1- and 2-celled embryos from the hybrid-inbred strain (DBA × C57B1/6) develop into morulae and blastocysts in vitro in either the complex medium, Ham's F-10, or the simple medium, BWW + BSA. In contrast, zygotes and early 2-celled embryos from the random-bred Swiss strain, CD-1, develop into morulae and blastocysts in only the simple medium, BWW + BSA, and do not progress past the 2-celled stage in the complex medium, Ham's F-10. The complex medium is not toxic to the random-bred embryos at later stages, as evidenced by the development to blastocysts of ≥ 50% of late 2-celled and 4-celled CD-1 embryos.

The evidence from the reconstitution experiments ("mock" Ham's F-10) showed that the inhibition of the second cleavage of the random-bred embryos was accomplished by relatively low concentrations of hypoxanthine. Hypoxanthine has also been implicated in inhibition of rabbit embryo development from the 2- to 4-cell stages in vitro (Kane and Foote, 1970), and mouse oocyte meiosis has been reported to be blocked by hypoxanthine in vitro (Downs et al., 1985; 1986) at the concentrations (2–5 mM) detected in follicular fluids in vivo (Eppig et al., 1985). These results suggest it may be necessary to re-evaluate the widespread use of Ham's F-10 in human and other animal studies (Wright et al., 1976; Edwards et al., 1980; Trounson et al., 1981; Ackerman et al., 1983; Lopata et al., 1982; Laufer et al., 1984; Quigley, 1985; Veeck, 1985).

The breeding experiments by us (Table 3) and others (Goddard and Pratt, 1983) show that the "2-cell block" of the random-bred embryos is not influenced by the paternal genome, and agree with the observations that early cleavage events are maternally directed (MacLaren, 1981). It is possible that the hypoxanthine is affecting a necessary maternal enzyme system that is expressed in lesser amounts in the random-bred embryos than in the hybrid-inbred embryos. This possibility is supported by the reported reversal of the "2-cell block" that follows microinjection of zygotes from a blocking strain with cytoplasm from zygotes of a nonblocking strain, indicating that small aliquots of enzymes or enzyme mRNAs are sufficient to overcome the block (Muggleton-Harris et al., 1982). If the oocyte is deficient in hypoxanthine-guanine phosphoribosyl transferase, the hypoxanthine can cause an increased synthesis of uric acid via the xanthine oxidase pathway. Mouse oocytes have recently been reported to convert radiolabeled hypoxanthine to uric acid (Eppig et al., 1985). However, although increased uric acid concentrations within the blastomeres could be toxic, the effect might not be a precise block at the second cleavage. Another enzyme system possibly affected by hypoxanthine is phosphodiesterase, an enzyme involved in the reduction of cyclic adenosine 3′,5′-monophosphate cell cleavage (Oleshansky, 1980). This possibility gains support from the preciseness of the block, and from the evidence for a similar role for hypoxanthine in inhibiting meiosis in mouse oocytes (Downs et al., 1985, 1986; Eppig et al., 1985).

CD-1 embryos have been previously reported to block at the 2-cell stage in simple culture medium (Biggers et al., 1968; Biggers, 1971). However, improved development of similar random-bred embryos (ICR) was reported when EDTA was added to the simple culture medium (Abramczuk et al., 1977), implying that the removal of trace metal ions was responsible for the progression of 48% of the pronuclear stage embryos to blastocysts. Metal ions other than those contained in the Ham's F-10 (Fe^{++}, Cu^{++}, and Zn^{++}) must have been responsible for the inhibition, however, since these were not found to be inhibitory in these studies (Table 4). The development to blastocysts of 39% of the random-bred zygotes in our BWW + BSA may also reflect removal of trace metal ions from the highly purified water

(redistilled, Type I, 18 megaohm) used to prepare our medium. The same water was used to prepare the Ham's F-10, which did not support development of the random-bred zygotes beyond the 2-cell stage, however, indicating that the "2-cell block" imposed by hypoxanthine on the random-bred embryos described in this report may not necessarily be due to inhibition of the same embryo functions involved in earlier reports of "2-cell block" mouse embryo development (Biggers, 1971). This discovery of the effect of hypoxanthine on the second cleavage of mouse embryo development provides a useful probe for understanding one of the essential steps in early embryo development. It will now be possible to discover which enzyme system(s) are affected, and whether or not embryos of other strains and other species are similarly inhibited by hypoxanthine.

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