In Vitro Preimplantation Mouse Embryo Development with Incubation Temperatures of 37 and 39°C

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Embryos from two strains of mice were used to assess the effect of incubation temperature on pronuclear and two-cell development to the morula/blastocyst (M/B) stage. Embryos from B6D2F2 and B6SJLF1 strains were cultured in medium M16 at either 37 or 39°C until 120 hr post human chorionic gonadotropin (hCG) or 0, 24, or 48 hr at 37°C and the remaining time at 39°C. Overall M/B development for pronuclear embryos was 0.6, 0, 32.3, and 52.4% for 0–96, 24–72, 48–48, and 96–0 hr at 37 and 39°C, respectively. Only 0–96 and 24–72 hr at 37 and 39°C were not different (P > 0.10). Overall M/B development for two-cell embryos was 48.1, 78.1, and 98.0% for 0–72, 24–48, and 72–0 hr at 37 and 39°C, respectively. Percentage development at each time was different (P < .01) for each category. Additionally, the number of nuclei for morulae and blastocysts tended to be higher for embryos initiating culture at the two-cell stage compared to pronuclear embryos. The first cell cycle was most dramatically affected by a 2°C increase in incubator temperature. More advanced embryos can tolerate slight increases in incubator temperature more readily than pronuclear embryos.

KEY WORDS: embryo culture; incubation temperatures.

INTRODUCTION

In vivo studies with mice have shown that maternal heat stress during the first days of gestation reduced embryo viability (15 to 20%) and subsequent litter size (100%) (1–3). Mice exposed to 34°C for 24 hr late in the day of mating had an increase in rectal temperature of 2°C. When embryos were flushed on day 1 postcoitum and incubated at 37°C for 2 days, the heat-stressed embryos yielded 17% fewer blastocysts than controls. Fewer blastocysts from heat-stressed females hatched compared to those from controls during the incubation period (2). Brinster (4) suggested that 37 to 37.5°C were optimal incubation temperatures for mouse embryos during in vitro culture studies. Few experiments have been conducted to evaluate the effect of in vitro culture temperature on the survival of mammalian embryos (see Rev. 5). In vitro maturation (IVM) and in vitro fertilization (IVF) of bovine oocytes are temperature dependent, showing the importance of temperature on critical phases of reproductive function (6). The in vivo heat stress (1,2) and in vitro temperature-dependent optimization of IVM and IVF (6) suggest that both maternal and paternal systems can be depressed by high ambient temperatures. The objective of this study was to assess the effect of incubation temperature on mouse embryo development to the blastocyst stage.

MATERIALS AND METHODS

Collection of Embryos

Three- to six-week-old B6D2F1 ($\bar{X}$ = 4.8 ± 1.3-week) and C57BL/6 ($\bar{X}$ = 4.7 ± 0.8-week) females were superovulated with intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG; Folligon, Intervet Laboratories Ltd., Cambridge, UK) at 1500 hr, followed 48 hr later by 2.5 IU human chorionic gonadotropin (hCG; Chorulon, Intervet Laboratories Ltd., Cambridge, UK). B6D2F1 females (C57BL/6 females × DBA/2 males; Harlan Olac, UK) were placed with B6D2F1 males overnight. C57BL/6 females were placed with SJL males overnight. One-cell embryos were ob-

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tained from excised oviducts at 20 to 22 hr after hCG (Day 1) in M2 medium (7) by dissecting the ampulla of each oviduct with a sterile dissecting scalpel. Ova within the cumulus mass released in to the M2 medium were exposed to hyaluronidase (Sigma Chemical Co., St. Louis, MO) prior to being placed into a washing well with fresh M2 medium (2.2-ml volume, four-well dishes; Nunclon, Medlabs Ltd., Dublin). Embryos from all mice of each strain ($\bar{X}$ = 32.4 for B6D2F1, n = 28, and $\bar{X}$ = 26.4 for C57BL/6, n = 30) on any particular day were first pooled in M2 medium and then washed in M16 medium (8) before allocation to treatment.

Two-cell embryos ($\bar{X}$ = 26.5 for B6D2F1 females, n = 30, and $\bar{X}$ = 23.0 for C57BL/6 females, n = 32) were recovered by dissection of oviducts in M2 media at 42 to 44 hr after hCG injection (9). The embryos were then washed first in M2 and then in M16 medium prior to allocation to culture in M16. All solutions, dishes, and instruments were maintained at 37 to 39°C before use.

Culture Media

All media were prepared fresh on the day of one-cell embryo recovery. All components were dissolved in Milli-Q water (Elga Ltd., High Wycombe Buck, UK) and bovine serum albumin was added last. The pH of the media was adjusted to 7.3 prior to final sterilization by filtration (0.20-μm filter, Acrodisc; Gelman Sciences, Ann Arbor, MI). The basic culture media were M2 (7) for collection and M16 (8) for short-term culture.

Culture Procedures

All cultures were performed in four-well dishes. The medium in each well (1 ml) was overlaid with 0.2 ml paraffin oil (Merck, Darmstadt, DDR). Media were equilibrated with 5% CO_2 in a sealed chamber (Jouan, S.A., Paris, France) at 37 or 39°C. One incubator (5) was used to assess temperature effects on development to the blastocyst stage of one- and two-cell embryos. The temperature was set at 37 or 39°C and remained at these temperatures for the duration of culture (96 hr for one-cell embryos and 72 hr for two-cell embryos) for the initial trials. Later on, temperature was maintained at 37°C for either 24 or 48 hr and then raised to 39°C for the duration of the culture period. Cultures were terminated after 96 hr (120 hr post hCG) and only morulae and blastocyst were fixed and stained for subsequent counting of nuclei. Morulae and blastocysts were fixed with ethyl alcohol:acetic acid (3:1) and stained with orcein acetic (BDH Chemicals Ltd., Poole, UK). Nuclei of abnormal embryos, which included those with degenerating and/or fragmenting blastomeres, were not counted. The classification systems of Chatot *et al.* (10) and Loutradis *et al.* (11) were used for assessment of embryo development.

Statistical Procedures

Chi-square analysis was used to test for treatment effects. Student's t test was used to assess differences in number of nuclei across treatment.

RESULTS

Development of pronuclear embryos at 37 and 39°C incubation temperatures is shown in Table I. Eighty-seven and seventy-seven percent of pronuclear embryos at 37 and 39°C were at the two-cell stage at 24 hr of incubation for B6D2F2 embryos. Fifty-eight and eighty-nine percent of embryos were two-cell embryos recovered from B6D2F1 females at 46 hr post hCG. The B6SJLF1 ova apparently did not fertilize as well, and two-cell comparisons at comparable times were generally lower. For B6D2F2 pronuclear embryos the percentage of two-cell embryos that reached the morula/blastocyst (M/B) stage was higher at 37°C (56%) than at 39°C (1%; $P < 0.01$). A similar pattern of development ($P < 0.01$) was found for B6SJLF1 embryos cultured at 37°C (41% M/B) and 39°C (0% M/B). The percentages M/B across mouse strains at 37 and 39°C for pronuclear embryos were not different (56 vs 41% for B6D2F2 vs B6SJLF1 at 37°C and 1 vs 0%, respectively, at 39°C). Pooling strains resulted in 52% M/B at 37°C, compared to 1% M/B at 39°C ($P < 0.01$).

Recovered B6D2F2 two-cell embryos had a higher percentage M/B at incubation temperatures of 37°C (98%) than at 39°C (76%; $P < 0.01$; Table I). Additionally, more ($P < 0.01$) B6D2F2 two-cell embryos attained M/B at 37°C (98%) than pronuclear embryos (56%) at 37°C. There was a higher percentage of M/B from two-cell embryos at 39°C (76%) than pronuclear embryos cultured at 39°C (1%; $P < 0.01$). A similar percentage M/B was found for both strains (98 vs 100%, respectively) developing from two-cell embryos at 37°C. However, two-cell embryos from B6D2F1 mice had a higher percentage (76%) M/B ($P < 0.01$) at 39°C than two-cell embryos

Table I. Percentage Development of Pronuclear and Two-Cell Mouse Embryos at 37 and 39°C at 120 hr Post hCG[a]

		Pronuclear embryos					Two-cell embryos				
Strain	Temperature	*n*	% 2-cell at 24 hr of incubation	M[b]	B[b]	M + B	*n*	% 2-cell at recovery	M	B	M + B
B6D2F2	37°C	269	87	14	42	56[c,*]	244	58	9	89	98[c]
	39°C	127	77	1	0	1[d]	64	89	6	70	76[de]
B6SJLF1	37°C	126	57	24	17	41[c]	116	48	9	91	100[c]
	39°C	245	23	0	0	0[d]	237	33	14	35	49[df]
Pooled	37°C	395	78	16	36	52[c]	360	55	9	89	98[c]
	39°C	372	42	1	0	1[d]	301	45	11	50	61[d]

[a] Percentage development at 120 hr post hCG (96 hr of incubation for pronuclear embryos and 72 hr of incubation for two-cell embryos). Values are percentages of two-cell-stage ova at 46 hr post hCG.
[b] M, morula; B, blastocyst.
* (c, d) Different superscripts within strain within initial stage of collection classifications differ (chi-square), $P < 0.01$; same superscripts between strain classifications do not differ (chi-square), $P > 0.10$. (e, f) Strains differ (chi-square), $P < 0.01$.

from C57BL/6 × SJL mice (49%). Percentages of M/B were greater ($P < 0.01$) for two-cell embryos than pronuclear embryos at either temperature (Table I). While the percentage M/B was higher ($P < 0.01$) for two-cell pooled strain embryos at 37°C (98%) than two-cell embryos at 39°C (61%) or pronuclear embryos at either temperature, there was no difference ($P > 0.10$) in the percentage M/B between pronuclear embryos cultured at 37°C (52%) and two-cell embryos cultured at 39°C (61%).

Numbers of nuclei for morulae and blastocysts of each mouse strain at each incubation temperature (37 vs 39°C) are given in Table II. Morulae from B6D2F1 matings had higher ($P < 0.05$) nuclei counts when two-cell embryos were cultured at 37°C than either pronuclear embryos cultured at 37°C or two-cell embryos cultured at 39°C (29 vs 24 and 27, respectively). Pronuclear embryos did not develop as well as two-cell embryos at either temperature. This is in contrast to morulae development of B6SJLF1 embryos, where no differences ($P > 0.10$) were found in nuclei numbers by the initial incubation stage of development or temperature. There were more ($P < 0.05$) nuclei in blastocysts from two-cell embryos (~61) at either temperature in B6D2F2 embryos than pronuclear embryos at 37°C (51). A similar pattern was found for B6SJLF1 embryos except that nuclei numbers in two-cell embryos incubated at 37°C (60) were greater ($P < 0.05$) than nuclei numbers in blastocysts from incubations at 39°C (44). Strain differences were apparent ($P < 0.05$) for number of nuclei in morulae cultured at 37°C but not at 39°C. B6D2F2 blastocysts had more ($P < 0.05$) nuclei than B6SJLF1 blastocysts at the end of culture when pronuclear embryos were incubated at 37°C and two-cell embryos incubated at 39°C but not ($P < 0.10$) when two-cell embryos were incubated at 37°C.

Development of pronuclear embryos to the two-cell stage at 37°C–24 hr and 37°C–48 hr for B6D2F2

Table II. Number of Nuclei (±SE; *n*) for Morulae and Blastocysts After Incubation of Pronuclear and Two-Cell Embryos at 37 or 39°C to 120 hr Post hCG

	Incubation temperature			
	37°C		39°C	
Morphological classification	Pronuclear embryos	2-cell embryos	Pronuclear embryos	2-cell embryos
		B6D2F2		
Morulae	23.8 ± 1.5 (14)[ac,*]	29.4 ± 1.9 (13)[bce]	—	26.8 ± 3.3 (4)[ce]
Blastocysts	50.9 ± 1.5 (42)[ae]	61.6 ± 1.5 (60)[bce]	—	61.1 ± 3.2 (22)[ce]
		B6SJLF1		
Morulae	19.6 ± 1.4 (14)[af]	18.5 ± 1.6 (12)[af]	—	20.0 ± 1.3 (9)[ae]
Blastocysts	41.5 ± 2.7 (8)[af]	59.8 ± 2.1 (31)[be]	—	43.7 ± 2.2 (12)[af]

* (a, b, c) Different superscripts within rows differ at $P < 0.05$. (e, f) Different superscripts within classification within columns differ at $P < 0.05$.

embryos was 58 and 68%, respectively (Table III). A similar pattern of two-cell embryo development was found for B6SJLF1 embryos at comparable incubation temperatures. Pronuclear embryos were extremely sensitive to the amount of time at 37 and 39°C and the 2°C increase in incubation temperature. The first 24 hr in culture, during which the first cell cycle occurs, appears to be the most sensitive, as 0% M/B were found when embryos were incubated at 37°C–24 hr and 39°C–72 hr. Increasing the amount of time for pronuclear embryos to 37°C–48 hr increased the percentage M/B to 32%. There were strain differences ($P < 0.01$) in percentage M/B development when pronuclear embryos were incubated at 37°C–48 hr and at 39°C–48 hr.

Development of two-cell embryos to M/B at 37°C–0 hr, 39°C–72 hr was lower ($P < 0.01$) than at 37°C–24 hr, 39°C–48 hr (33 vs 68% for B6D2F2 embryos and 51 vs 100% for B6SJLF1 embryos; Table III). Within strains the greater amount of time at 39°C led to a significant ($P < 0.01$) lower percentage M/B formation at the end of culture. The B6SJLF1 embryo development of M/B was higher (100%, $P < 0.01$) than B6D2F2 embryo development (68%). When data were pooled across strains the additional 24 hr at 37°C resulted in a 36% increase ($P < 0.01$) in M/B development by the end of the culture period.

Numbers of nuclei were higher ($P < 0.05$) for two-cell embryos which reached the morula stage of development for B6D2F2 embryos cultured at 37°C–24 hr, 39°C–48 hr (26) than for pronuclear embryos under conditions of 37°C–48 hr, 39°C–48 hr embryos (20) (Table IV). Additionally, the only strain difference ($P < 0.05$) was between B6D2F2 pronuclear embryos and B6SJLF1 pronuclear embryos, which reached the morula stage of development at 37°C–48 hr, 39°C–48 hr (20 vs 26).

A summary of the pronuclear and two-cell embryo development to the M/B stage is given in Table V. Culture of pronuclear embryos for 0 to 24 hr at 37°C resulted in essentially no development to the M/B stages. Culture of pronuclear embryos for 48 and 96 hr at 37°C led to significant increases ($P < 0.01$) in M/B development (32 and 52%, respectively). Two-cell embryos tolerated 39°C incubation conditions better than pronuclear embryos, with 48, 78, and 98% M/B formation at 72, 48, and 0 hr at 39°C, respectively. Thus it appears that the first cell cycle is most detrimentally affected by high incubator temperature. More advanced embryos appear to be able to tolerate the 2°C increase in incubator temperatures.

DISCUSSION

The percentage development of B6D2F2 pronuclear embryos to the two-cell stage at 24 hr in culture at either 37 or 39°C was comparable to that in

Table III. Percentage Development of Pronuclear and Two-Cell Mouse Embryos at 37°C for 0, 24, or 48 hr and at 39°C for 72 or 48 hr at 120 hr Post hCG[a]

		Pronuclear embryos						Two-cell embryos				
Strain	Temperature and time	*n*	% 2-cell at 24 hr of incubation	M[b]	B[b]	M + B	Time	*n*	% 2-cell at recovery	M	B	M + B
B6D2F2	37°C–24 hr 39°C–72 hr	136	58	0	0	0[c,*]	0 hr 72 hr	142	98	6	27	33[c]
	37°C–48 hr 39°C–48 hr	201	68	21	21	42[de]	24 hr 48 hr	187	84	10	58	68[de]
B6SJLF1	37°C–24 hr 39°C–72 hr	199	61	0	0	0[c]	0 hr 72 hr	176	71	6	45	51[c]
	37°C–48 hr 39°C–48 hr	109	51	7	2	9[df]	24 hr 48 hr	131	58	8	92	100[df]
Pooled	37°C–24 hr 39°C–72 hr	335	60	0	0	0[c]	0 hr 72 hr	318	83	6	36	42[c]
	37°C–48 hr 39°C–48 hr	310	62	17	15	32[d]	24 hr 48 hr	318	73	9	69	78[d]

[a] Percentage development at 120 hr post hCG (96 hr of incubation for pronuclear embryos and 72 hr of incubation for two-cell embryos). Values are percentages of two-cell embryos at 46 hr post hCG.

[b] M, morula; B, blastocyst.

* (c, d) Different superscripts within strain within initial stage of collection classification differ (chi-square), $P < 0.01$; same superscripts between strain classifications do not differ (chi-square), $P > 0.10$. (e, f) Strains differ (chi-square), $P < 0.01$.

Table IV. Number of Nuclei (±SE; *n*) for Morulae and Blastocysts After Incubation of Pronuclear and Two-Cell Embryos for Various Times at 37 and/or 39°C

	Incubation temperature and time			
	Pronuclear embryos		Two-cell embryos	
Morphological classification	37°C–24 hr 39°C–72 hr	37°C–48 hr 39°C–48 hr	37°C–0 hr 39°C–72 hr	37°C–24 hr 39°C–48 hr
		B6D2F2		
Morulae	—	20.1 ± 0.8 (19)^{ae,*}	21.8 ± 1.5 (5)^{abe}	25.6 ± 1.8 (11)^{bce}
Blastocysts	—	55.7 ± 3.6 (12)^{a}	60.0 ± 2.4 (22)^{ae}	59.7 ± 2.1 (46)^{ae}
		B6SJLF1		
Morulae	—	26.0 ± 1.2 (3)^{af}	23.3 ± 1.4 (6)^{ae}	28.2 ± 4.3 (6)^{ae}
Blastocysts	—	—	61.9 ± 2.9 (22)^{ae}	66.3 ± 1.6 (41)^{ae}

* (a, b, c) Different superscripts within row differ at $P < 0.05$. (e, f) Different superscripts within classification within columns differ at $P < 0.05$.

reports of other reachers (11–13). The overall percentage of pronuclear embryo development to the two-cell stage at 24 hr for B6SJLF1 embryos was less than that for B6D2F2 embryos. Moreover, the rate of blastocyst formation at 37°C for this strain was less than that reported by Whitten and Biggers (14) for embryos from C57BL/10 × SJL matings. They found that differences in blastocyst development were dependent upon mouse strain. Recently Gardner and Leese (15) reported 82% development of one-cell B6CBAF1 embryos in M16 medium. Additionally, development of two-cell B6D2F2 embryos at 37°C to the blastocyst stage was comparable to that reported by Loutradis *et al.* (11) for the same strain.

It was apparent that pronuclear embryos do not develop at 39°C, while a significant reduction in M/B development occurs when two-cell embryos are exposed to the same incubator temperature. The effect of a 2°C elevation in incubation temperature on pronuclear and two-cell embryos supports *in vivo* heat-stress studies (1–3). It appears that the effect of heat stress on early embryo development is a mechanical one mediated by elevated body temperature in vivo rather than through physiological or endocrine mechanisms of maternal origin because our in vitro study maintained constant conditions, with only a change in incubation temperature. While heat-shock proteins are thought to protect organisms from deleterious effects of heat (16), the secretions of significant amounts of protein (17) and, specifically, heat-shock proteins (16) do not occur until the morula/blastocyst stage of development. Baumgartner and Chrisman (18) suggest that embryonic losses caused by heat were due to chromosomal abberations or disrupted metabolic activity.

Twenty-four hours at 37°C was not sufficient time to overcome the deleterious effects of the 39°C incubation environment for pronuclear embryos. Forty-eight hours at 37°C provided some protection from the elevated temperature, as 32% of these embryos attained the M/B stage of development in vitro. Two-cell embryos were less susceptible to elevated incubator temperatures. Elliott and Ulberg (3) observed greater percentages of two- and four-cell embryos than eight-cell embryos at 51 hr postcoitum, when a 32 to 34°C environmental heat stress, compared to a 21°C environment, was imposed for 24 hr late on the day of vaginal plug observation. This is consistent with our in vitro results.

Table V. Percentage of Morula and Blastocyst Development of Pronuclear and Two-Cell Embryos Incubated for Various Times at 37 and 39°C[a]

Hours at		
37°C	39°C	
		Pronuclear embryos
0	96	1/155 = 0.6%^{b,*}
24	72	0/201 = 0%^{b}
48	48	62/192 = 32.3%^{c}
96	0	161/307 = 52.4%^{d}
		Two-cell embryos
0	72	192/399 = 48.1%^{b}
24	48	182/233 = 78.1%^{c}
72	0	193/197 = 98.0%^{d}

[a] Combined percentage development for B6D2F2 and B6SJLF1 strains at 120 hr post hCG (96 hr of incubation for pronuclear embryos and 72 hr of incubation for two-cell embryos) for percentage of two-cell stage embryos at 46 hr post hCG.

* (b, c, d) Different superscripts within classification differ (chi-square), $P < 0.01$.

Our results contrast with those of Lavy *et al.* (19), who used a different strain (B3G6F1) of mice and a constant amount of time of elevated incubator temperature for both pronuclear and two-cell embryo culture. At the same stages of embryo development (48 hr of incubation for two-cell embryos and 72 hr for pronuclear embryos), they found 83% M/B at 37°C and 95% M/B at 39°C from two-cell embryos, compared to 49% morulae at 37°C and 29% morulae at 39°C from pronuclear embryos. Blastocyst development did not occur from pronuclear embryos at 72 hr in culture and agrees with our results (unpublished observations). Therefore their lack of incubator effect on pronuclear embryo development could not be tested completely, although their data tend to suggest that the temperature effect is apparent for pronuclear embryos, as in our study.

Nuclei counts for blastocysts in our study averaged slightly higher than those of Gardner and Leese (15) but are within the range of what they reported ($\bar{X}$ = 45 to 46, range = 28 to 80). Estimates of all numbers for both morulae and blastocysts are considerably higher than that reported by Chatot *et al.* (10) and may be due to strain differences in nuclei numbers at the blastocyst stage of development (20). Two-cell embryos generally had higher morula and blastocyst cell numbers than pronuclear embryos, which suggests a delay in development for the earlier-stage embryos when cultured in vitro to the same end point. The strain difference in nuclei tends to support the morphological changes observed and suggests less viability for the B6SJLF1 embryos compared to the B6D2F2 embryos. It is not unreasonable to believe that strain differences exist with in vitro embryo development, as they have been reported in mice (14,20) and tolerance to heat stress is different for breeds/strains of other mammals.

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