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Foetal and Placental Growth in the Mouse after Pre-implantation Development *in vitro* under Oxygen Concentrations of 5 and 20%

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Abstract

Blastocysts which developed from two-cell mouse embryos in culture tubes containing an atmosphere with 20% oxygen had approximately 20% fewer blastomeres than blastocysts which developed under an oxygen concentration of 5%. When these smaller blastocysts were transferred to the uteri of pseudopregnant foster mothers, the foetuses developing were as viable as those developing from blastocysts cultured under 5% oxygen, indicating their ability to regulate for a lower blastomere number by at least day 17 of development.

The transfer operation itself had no adverse effect on foetal or placental growth. However, culture of blastocysts *in vitro* did depress foetal though not placental growth, suggesting that the inner cell mass is more susceptible than the trophectoderm to culture *in vitro*. Foetal but not placental growth was lower following the transfer of blastocysts to a day-3 rather than a day-4 uterus.

Four cases of placental fusion were found. In one case, the foetuses were contained within the same embryonic sac and may have been twins.

Introduction

When mouse embryos are cultured to the blastocyst stage using an atmosphere of 10% CO_2 in air, their viability and postimplantational growth after transfer to foster mothers are lower than those of control, uterine blastocysts (Bowman and McLaren 1970*a*). However, the optimal oxygen concentration for development of pre-implantation mouse embryos to the blastocyst stage *in vitro* is between 2·5 and 5% (Whitten 1971; Quinn and Harlow 1978). It is possible that viability and postimplantational growth of blastocysts might be improved if they were cultured under the optimal oxygen concentration.

Blastocysts cultured under 20% O_2 have fewer blastomeres than those developing under 5% O_2 (Quinn and Harlow 1978). Thus cultured blastocysts with different blastomere numbers can be tested for their ability to attain normal embryonic size. Where 'large' blastocysts are produced by fusing two eight-cell embryos, size regulation occurs during or shortly after implantation (Bowman and McLaren 1970*a*; Buehr and McLaren 1974).

The slowing of cell division in blastocysts cultured under 20% O_2 rather than 5% O_2 may be occurring differentially in the inner cell mass which has been shown to be more susceptible than the trophectoderm to treatments using [^{3}H]thymidine (Snow 1973) and X-rays (Goldstein *et al.* 1975). If this were the case and no regulation for small size occurred, then a decreased foetal weight but not placental weight would be observed after the transfer of blastocysts cultured under 20% O_2 to foster mothers.

In the present study the technique of embryo transfer has been used to investigate (1) whether the viability and postimplantational growth of cultured mouse embryos can be improved by incubation under 5% O_2 rather than 20% O_2, (2) whether blastocysts with fewer blastomeres can regulate for their small size, and (3) whether the slowing of cell division in small blastocysts occurs differentially in their inner cell mass.

Materials and Methods

Embryo Culture

All mice used were of the F_1 hybrid cross (C57BL×CBA, or reciprocal). Virgin females aged 3–5 weeks were superovulated with 10 i.u. each of pregnant mare serum gonadoptrophin and human chorionic gonadotrophin (Folligon and Chorulon, respectively, Organon Laboratories) given 46–50 h apart, after which the females were placed with fertile males. Two-cell embryos were collected on day 2 of pregnancy (day of vaginal plug = day 1) and cultured for 70–74 h in tubes which were gassed with atmospheres of 5% O_2–5% CO_2–90% N_2 or 20% O_2–5% CO_2–75% N_2 as described by Quinn and Harlow (1978). The percentage of embryos developing to the blastocyst stage was recorded. The number of blastomeres was assessed in some blastocysts using an air-drying technique based on that of Tarkowski (1966).

Embryo Transfer and Assessment of Development

Blastocysts were transplanted to the uteri of day-3 or day-4 pseudopregnant recipients which had mated with sterility-tested vasectomized males (day of plug = day 1). The transfer operation was similar to that described by Mullen and Carter (1973). Four to six blastocysts were transferred to each uterine horn, one horn receiving blastocysts cultured under 5% O_2, the other receiving blastocysts cultured under 20% O_2. The horns used for each treatment were randomly alternated among the transfers. Some freshly collected day-4 embryos were transferred to the uteri of day-4 pseudopregnant recipients. As a further control, the litters of some pregnant mice were allowed to develop entirely *in vivo*.

Recipients were killed by cervical dislocation 14 days after the transfer operation. Control females (unoperated) were killed on days 16, 17 or 18 of gestation. Uteri were removed and the number of foetuses and resorptions was recorded. Each foetus and placenta was removed from its membranes, separated from the umbilical cord, blotted on filter paper and weighed. Foetal crown–rump length was recorded. The developmental stage of the foetuses was assessed according to the morphological rating scale described by Walker and Crain (1960).

Statistical Methods

Comparison of the effects of 5 and 20% O_2 on responses was tested by Student's *t*-test. The percentages of transferred blastocysts implanting and developing were transformed to angles before analysis. Foetal and placental growth were analysed on a weighted uterine horn difference as described by Bowman and McLaren (1970*a*). The effect of litter size on foetal and placental weight was assessed by linear regression analysis after the data were transformed to logarithms.

Results

Blastocyst development from two-cell embryos *in vitro* under oxygen concentrations of 5 and 20% is given in Table 1. Approximately 90% of the embryos developed to the blastocyst stage over 3 days of culture under both 5 and 20% O_2. Blastocysts which developed under 20% O_2, however, had significantly fewer blastomeres than those which developed under 5% O_2 ($t_{26} = 2{\cdot}4$, $P < 0{\cdot}05$). Freshly collected day-4 embryos contained the same number of blastomeres as blastocysts developing under 5% O_2, although they were 24 h younger than the cultured blastocysts.

Table 2 gives the results for the implantation and development of embryos from transferred blastocysts. Most (90%) of the recipient females became pregnant.

Those not becoming pregnant had received cultured blastocysts on day 4 of pseudopregnancy. Blastocysts which developed under 5 or 20% O_2 *in vitro* were equally able to implant and develop into live foetuses ($t_{20} = 0{\cdot}32$, $P > 0{\cdot}5$). There was no

Table 1. Blastocyst development *in vitro* under oxygen concentrations of 5 and 20%
Values shown are means ±s.e., with the number of replications in parentheses

	Oxygen concentration		Freshly collected day-4 embryos
	5%	20%	
% 2-cells forming blastocysts[A]	91·0±2·1 (7)	87·7±2·9 (7)	—
No. of blastomeres per blastocyst	50·6±4·3 (12)	41·3±2·7 (16)	50·5±3·5 (12)

[A] 30–50 embryos per replicate.

significant difference between transferring blastocysts to recipients on day 3 or day 4 of pseudopregnancy with respect to the number of embryos implanting ($t_9 = 0{\cdot}86$, $P > 0{\cdot}2$) or the number developing for 14 days *in utero* ($t_9 = 2{\cdot}14$, $P > 0{\cdot}05$).

Table 2. Implantation and development of embryos after transfer of blastocysts to pseudopregnant hosts

	Freshly collected day-4 blastocysts	Blastocysts cultured for 70–74 h from the 2-cell stage			
Day of pseudopregnancy of recipient	Day 4	Day 4		Day 3	
No. of recipients	4	7		6	
No. becoming pregnant	4	5		6	
		Oxygen concentration			
	—	5%	20%	5%	20%
No. of blastocysts transferred to recipients becoming pregnant	37	25	26	35	34
Total No. of embryos implanting	11	15	16	24	24
% Transferred embryos implanting per mouse (mean±s.e.)	30±13	57±16	64±10	70±15	71±13
Total No. of embryos alive at autopsy	9	5	5	12	10
% Transferred embryos developing per mouse (mean±s.e.)	25±14	19±5	20±11	36±11	29±10

Mean foetal and placental weights, foetal length and morphological rating were calculated for each naturally pregnant female (*in vivo* control) or for each horn of recipient females. No effect of litter size on foetal or placental weight was revealed by regression analysis in naturally pregnant females (average litter size = 8·6) nor recipient females receiving cultured blastocysts (average litter size = 3·8). The data, therefore, have not been weighted for litter size. Foetal and placental growth are shown in Fig. 1. Although blastocysts cultured under 20% O_2 had fewer blastomeres

than those cultured under 5% O_2 (Table 1), they attained the same foetal weight (Fig. 1*a*; $t_4 = 0{\cdot}37$, $P > 0{\cdot}5$) and placental weight (Fig. 1*b*; $t_4 = 0{\cdot}06$, $P > 0{\cdot}5$) as the blastocysts cultured under 5% O_2. Foetuses developing from blastocysts transferred to a day-3 uterus were significantly lighter, shorter and less well developed

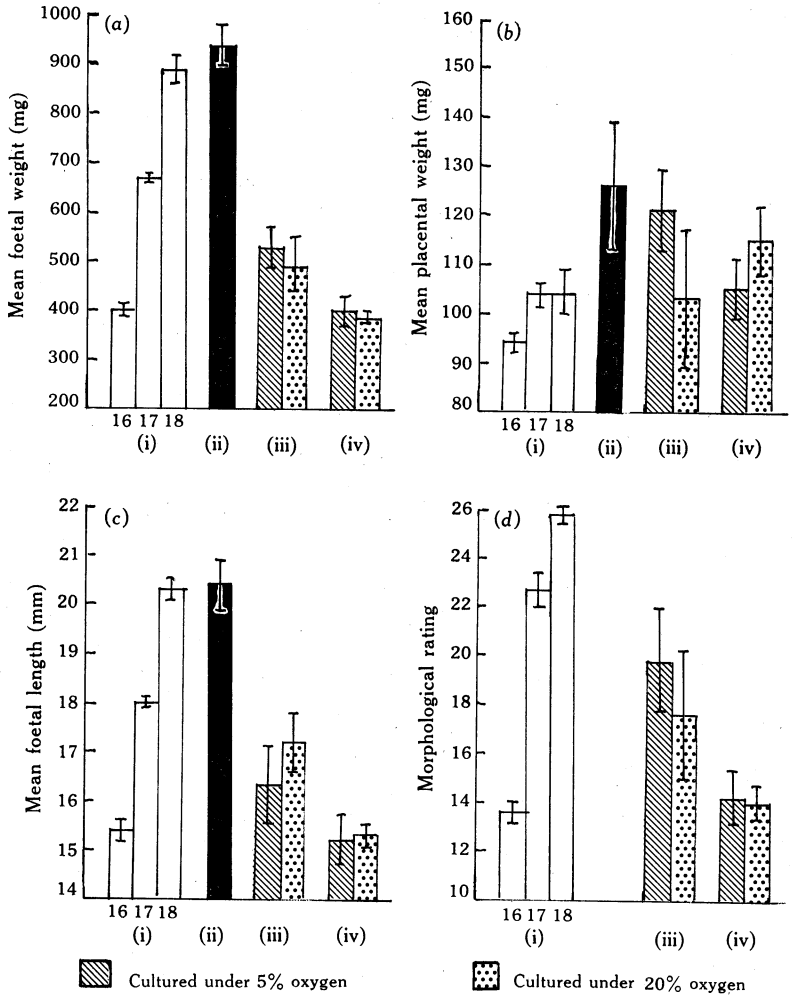

Fig. 1. Mean (±s.e.) foetal and placental growth after transfer of cultured embryos. (i) *In vivo* controls on days 16, 17 and 18; (ii) transfer of day-4 uterine blastocysts; (iii) transfer of cultured blastocysts to a day-4 uterus; (iv) transfer of cultured blastocysts to a day-3 uterus.

than those developing from blastocysts transferred to a day-4 uterus (Figs 1*a*, 1*c* and 1*d*; $t_{30} = 3{\cdot}1$, $t_{29} = 3{\cdot}1$, $t_{29} = 3{\cdot}1$ respectively; $P < 0{\cdot}01$). There was no significant difference, however, between the placental weights of these two groups of foetuses (Fig. 1*b*; $t_{30} = 0{\cdot}34$, $P > 0{\cdot}2$).

Foetal and placental growth following the transfer of freshly collected day-4 embryos was equivalent to the growth of day-18 foetuses developing entirely *in vivo*

(Figs 1*a*, 1*b* and 1*c*). This indicates that the transfer operation itself had no effect on foetal and placental growth. The weight of foetuses developing from cultured blastocysts was significantly lower (Fig. 1*a*; $t_{17} = 8{\cdot}1$, $P < 0{\cdot}001$; $t_{29} = 14{\cdot}0$, $P < 0{\cdot}001$ for those transferred to day-4 and day-3 uteri respectively) compared with that of freshly collected blastocysts. There was no significant difference between the day-4 uterine blastocysts and the cultured blastocysts in placental weight at autopsy (Fig. 1*b*; $t_{17} = 0{\cdot}86$, $P > 0{\cdot}2$; $t_{29} = 1{\cdot}4$, $P > 0{\cdot}1$ for those transferred to day-4 and day-3 uteri respectively). Overall, cultured blastocysts were retarded in development by 1½–2 days compared to their *in vivo* counterparts (Fig. 1*d*).

In this study, a foetus was considered to be a runt if it weighed less than two standard errors below its litter mean and was less than two morphological ratings below the litter mean in developmental stage. Runting occurred in 1·3% (4/300) of foetuses developing entirely *in vivo* and in 2·4% (1/41) of foetuses after transfer of cultured blastocysts. The incidence of placental fusion was 1·4% (3/211) in unoperated mice and 5·9% (1/17) in operated mice. This incidence is based on the number of 'placental neighbourships' as defined by McLaren and Michie (1959). In three cases of placental fusion (two developing *in vivo* and one from blastocysts cultured under 20% O_2), one foetus of each pair was alive with a weight similar to its litter mates. The other foetus of each pair was dead and had undergone some degree of resorption. In the other case of placental fusion, which occurred in an unoperated mouse, the foetal pair was within a single yolk sac, and both foetuses were alive with similar weights to each other although slightly smaller than their litter mates. These may have been twins.

Discussion

In the present study mouse blastocysts developing from two-cell embryos under an atmosphere containing 20% O_2 have approximately 20% fewer blastomeres than those which develop under 5% O_2. This is similar to the results obtained for this F_1 hybrid type (C57BL × CBA) by Quinn and Harlow (1978). The foetuses developing from these smaller blastocysts were able to regulate for their lower blastomere number by at least day 17 of development. The mechanism of this regulation is not known. Buehr and McLaren (1974) suggest that chimaeric double embryos retard their growth soon after implantation rather than accelerate their differentiation. Tarkowski (1959) claimed that regulation of 'half' embryos occurs between the 10th and 12th day of gestation and noted that this coincided with the functioning of the chorio–allantoic placenta which may permit an acceleration of growth. To establish when regulation for small size occurs, embryos should be examined at regular intervals in a manner similar to that described by Buehr and McLaren (1974) but extending the study to the whole period of gestation.

Transfers of cultured mouse blastocysts to a day-3 or day-4 uterus are asynchronous since cultured embryos are up to 24 h older than uterine embryos containing an equivalent cell number (Table 1; Bowman and McLaren 1970*b*). In the present study the different aged uteri were equally able to support implantation and development of transferred blastocysts. Other workers have also shown that more embryos implant and develop when transferred to a developmentally younger uterus (McLaren and Michie 1956; Aitken *et al.* 1977). The relative ages of the embryos and uterus may be more important than the specific age of the uterus in transfers. Foetuses developing from the transfer of blastocysts to a day-3 uterus were generally smaller

than those developing from blastocysts transferred to a day-4 uterus. Although asynchronous transfer may give more blastocysts the opportunity to implant while the uterus is reaching its state of maximum receptivity (McLaren and Michie 1956; Finn and Martin 1974), the delay in implantation is probably responsible for the lower foetal weight observed in our study. No difference in weight between placentae developing from blastocysts transferred to a day-3 or day-4 uterus was observed in the present work. The difference found in foetal but not placental weight occurs because the placenta achieves maximum weight and plateaus earlier than foetal weight (Fig. 1*b*; McLaren 1965*a*). The transfer operation in this study did not adversely affect foetal or placental growth. However, Aitken *et al.* (1977) claimed there was a 'transfer effect' which resulted in depressed weights. This difference could be due to the use of different types of mice (randomly bred *v.* hybrid) or to the operation procedure itself.

Culture of pre-implantation embryos *in vitro* depressed foetal growth, but not placental growth. A similar effect of culture was found by Bowman and McLaren (1970*a*), though they did not compare the weights with embryos developing *in vivo* where the effect of culture is even more pronounced (Fig. 1). Bowman and McLaren (1970*a*) suggested that the real effect of culture on foetal weight was greater than their values showed since McLaren (1965*b*) claimed that foetal weight was inversely related to the number of young in the litter. Thus, the smaller number of foetuses developing per horn from cultured blastocysts compared to control uterine blastocysts should allow foetuses from cultured blastocysts to gain a greater weight. No such relationship was found in the present work. The development of foetuses from cultured blastocysts was consistently retarded in the present study compared with that of foetuses developing entirely *in vivo*, despite the fact that the litter size was approximately halved. Also, foetuses from uterine blastocysts did not differ significantly in weight compared with day-18 foetuses developing entirely *in vivo* even though the average litter size was considerably lower (8·5 *v.* 2·3). This suggests that litter size in the type of mouse used in this study (C57BL × CBA, F_1) is not an important parameter in determining foetal weight. A similar conclusion was drawn by Aitken *et al.* (1977) for lines of mice selected for different body weights.

The suggestion that the inner cell mass is more sensitive to suboptimal culture conditions than the trophectoderm (Bowman and McLaren 1970*a*) is supported by the observations in this study since foetal weight was depressed but placental weight was unaffected whether cultured blastocysts were transferred to a day-3 or a day-4 uterus. Improving the culture conditions by culturing under the optimal oxygen level apparently does not overcome the depression of foetal growth. Any sensitivity of the inner cell mass to a slower rate of cell division under 20% O_2 compared to 5% O_2 is not apparent in this study, and if it was occurring, it would be masked by the ability of the smaller blastocyst to regulate for its size.

The incidence of runts in the present work was similar to that found by McLaren and Michie (1960). The incidence of placental fusion was also similar to that found by McLaren and Michie (1959). Hollander and Strong (1950), in a study on placental fusion, found no definite case of monozygotic twinning. However, in the present study two foetuses developing entirely *in vivo* shared the same foetal membranes as well as placenta. The origin of these 'twins' is unknown. They may have been genuine monozygotic twins or could have resulted from the fusion of trophoblast-derived tissue of two blastocysts occupying the same implantation site in the uterus. There

have been no previous reports of naturally occurring 'twins' in polytoccus mammalian species.

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References

Aitken, R. J., Bowman, P., and Gauld, I. (1977). The effect of asynchronous egg transfer on foetal weight in mice selected for large and small body size. *J. Embryol. Exp. Morphol.* **37**, 59–64.

Bowman, P., and McLaren, A. (1970*a*). Viability and growth of mouse embryos after *in vitro* culture and fusion. *J. Embryol. Exp. Morphol.* **23**, 693–704.

Bowman, P., and McLaren, A. (1970*b*). Cleavage rate of mouse embryos *in vivo* and *in vitro*. *J. Embryol. Exp. Morphol.* **24**, 203–7.

Buehr, M., and McLaren, A. (1974). Size regulation in chimaeric mouse embryos. *J. Embryol. Exp. Morphol.* **31**, 229–34.

Finn, C. A., and Martin, L. (1974). The control of implantation. *J. Reprod. Fertil.* **39**, 195–206.

Goldstein, L. S., Spindle, A. I., and Pederson, R. A. (1975). X-ray sensitivity of the preimplantation mouse embryo *in vitro*. *Radiat. Res.* **62**, 276–87.

Hollander, W. F., and Strong, L. C. (1950). Intra-uterine mortality and placental fusions in the mouse. *J. Exp. Zool.* **115**, 131–7.

McLaren, A. (1965*a*). Placental weight loss in late pregnancy. *J. Reprod. Fertil.* **9**, 343–6.

McLaren, A. (1965*b*). Genetic and environmental effects on foetal and placental growth in mice. *J. Reprod. Fertil.* **9**, 79–98.

McLaren, A., and Michie, D. (1956). Studies on the transfer of fertilized mouse eggs to uterine foster-mothers. *J. Exp. Biol.* **33**, 394–416.

McLaren, A., and Michie, D. (1959). Experimental studies on placental fusion in mice. *J. Exp. Zool.* **141**, 47–73.

McLaren, A., and Michie, D. (1960). In 'Ciba Foundation Symposium on Congenital Malformations'. (Eds G. E. W. Wolstenholme and C. M. O'Connor.) pp. 178–93. (Elsevier: Amsterdam.)

Mullen, R. J., and Carter, S. C. (1973). Efficiency of transplanting normal, zona-free, and chimeric embryos to one and both uterine horns of inbred and hybrid mice. *Biol. Reprod.* **9**, 111–15.

Quinn, P., and Harlow, G. M. (1978). The effect of oxygen on the development of preimplantation mouse embryos *in vitro*. *J. Exp. Zool.* **206**, 73–81.

Snow, M. H. L. (1973). Abnormal development of preimplantation mouse embryos grown *in vitro* with ^{3}H-thymidine. *J. Embryol. Exp. Morphol.* **29**, 601–15.

Tarkowski, A. K. (1959). Experimental studies on regulation in the development of isolated blastomeres of mouse eggs. *Acta Theriol.* **3**, 191–267.

Tarkowski, A. K. (1966). An air-drying method for chromosome preparation from mouse eggs. *Cytogenetics* **5**, 394–400.

Walker, B. E., and Crain, B. (1960). Effects of hypervitaminosis A on palate development in two strains of mice. *Am. J. Anat.* **107**, 49–58.

Whitten, W. K. (1971). Nutrient requirements for the culture of preimplantation embryos *in vitro*. *Adv. Biosci.* **6**, 129–41.

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